Development and evaluation of a recombinant vaccine against *Clostridium perfringens* type D epsilon toxin

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: …….. Date: ……..

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SUMMARY

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Enterotoxemia, an economically important disease of sheep, goats and calves, is caused by systemic effects of the epsilon toxin produced by the anaerobic bacterium *Clostridium perfringens* Type D. To combat the disease, a vaccine consisting of formalin-inactivated Type D toxin is available, but several concerns regarding its production have been raised. The development of efficacious recombinant subunit vaccines can provide a means whereby many of the production problems may be eliminated or minimized. However, the production of purified antigens is often associated with weakened antigen immunogenicity. New vaccine strategies therefore rely on the incorporation of effective adjuvants, which stimulate the innate immune response and, in turn, activate the adaptive immune response. An increasing number of studies have demonstrated that flagellin is a potent activator of a broad range of cell types involved in innate and adaptive immunity. Consequently, the aim of this investigation was to produce different recombinant vaccine candidates, inclusive of a flagellin-epsilon toxin fusion protein, for preventing Type D enterotoxemia.

Attempts at expressing the native gene sequence for the Type D epsilon toxin in *Escherichia coli* have been characterized by low levels of expression. This may be due to differences in

the codon bias between the heterologous gene and *E. coli.* Therefore, in this study, a completely synthetic codon-optimized gene encoding the epsilon toxin was obtained. The full-length *etx* gene (*etx*0), an *etx* gene lacking the first nine amino acids of the signal peptide sequence (*etx*1), as well as a flagellin-toxin fusion gene composed of the *etx*1 gene and a truncated *hag* gene of *Bacillus halodurans* Alk36 (Δ*hag::etx*1) were expressed in *E. coli* BL21-Gold(DE3) cells. Expression of the respective recombinant proteins, designated ETX0, ETX1 and NC-ETX1, respectively, were confirmed by Western blot analysis using anti-epsilon toxin antibodies. The ETX0 protein was expressed in a soluble form, whereas the ETX1 and NC-ETX1 proteins were insoluble and accumulated in inclusion bodies. In an attempt to optimize expression of the recombinant proteins, different cultivation media and concentrations of the promoter inducer were evaluated. Under optimized expression conditions, the yields of ETX0, ETX1 and NC-ETX1 were determined to be 828 mg/l, 395 mg/l and 525 mg/l, respectively.

Despite having expressed the recombinant proteins with an N-terminal hexahistidine tag, the recombinant ETX0 protein could not be purified with nickel (Ni^{2+}) chelate affinity chromatography. This was due to removal of the affinity tag as a consequence of intracellular processing of the N-terminal signal peptide sequence. The recombinant ETX0 protein was consequently purified to near homogeneity by ion exchange chromatography. The recombinant ETX1 and NC-ETX1 proteins were purified from inclusion bodies by affinity chromatography under denaturing conditions and then refolded in TBS buffer. The yield of purified ETX0, ETX1 and NC-ETX1 were determined to be 430 mg/l, 400 mg/l and 340 mg/l, respectively. The cytotoxicity of these purified recombinant proteins was assayed *in vitro* against Madin-Darby canine kidney (MDCK) cells, the results of which indicated that NC-ETX1 was not toxic towards the cells and both ETX0 and ETX1 displayed moderate toxicity towards the cells.

Cumulatively, the results indicated that the respective proteins were expressed to high levels in *E. coli* and large amounts of purified proteins could be obtained. These recombinant proteins can in future be evaluated as vaccine candidates against C. *perfringens* Type D enterotoxemia.

TABLE OF CONTENTS

DECLARATION **i**
ACKNOWLEDGEMENTS **ii**
SUMMARY **iii**
LIST OF ABBREVIATIONS **ix**
LIST OF FIGURES **xiii**
LIST OF TABLES **xv**

CHAPTER 1 **1**

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION 2
1.2 ENTEROTOXEMIA IN LIVESTOCK 3
1.2.1 Introduction to *Clostridium perfringens* Type D 3
1.2.2 *C. perfringens* Type D disease 5
1.2.3 Clinical symptoms 6
1.2.4 Pathogenesis 7
1.2.5 Diagnosis of enterotoxemia 9
1.3 THE EPSILON TOXIN OF *C. perfringens* Type D 10
1.3.1 Epsilon toxin genetics and production 10
1.3.2 Structure of the epsilon toxin 11
1.4 PREVENTION OF ENTEROTOXEMIA DISEASE 13
1.4.1 Epsilon toxoided vaccines 13
1.4.2 Recombinant epsilon vaccines 14
1.5 VACCINE ADJUVANTS 15
1.5.1 Innate and adaptive immunity 16
1.5.2 Adjuvants used in epsilon toxoided vaccines 18
1.5.3 Flagellin adjuvants 19
1.6 AIMS OF THIS STUDY 23

CHAPTER 2 **25**

Cloning and expression of *Clostridium perfringens* Type D epsilon toxins and flagellin-toxin fusion proteins in *Escherichia coli*

2.1 INTRODUCTION 26
2.2 MATERIALS AND METHODS 28
2.2.1 Bacterial strains, plasmids and culturing conditions 28
2.2.2 Genomic DNA extraction 30
2.2.3 DNA amplification 30
2.2.3.1 Primers 30
2.2.3.2 Polymerase chain reaction (PCR) 30
2.2.4 Agarose gel electrophoresis 32
2.2.5 Purification of DNA from agarose gels 32
2.2.6 Cloning of DNA fragments into plasmid vectors 32
2.2.7 Transformation of *E. coli* 33
2.2.7.1 Preparation of electro-competent *E. coli* cells 33
2.2.7.2 Electroporation 33
2.2.8 Screening and characterization of transformants 34
2.2.8.1 Plasmid DNA extraction 34
2.2.8.2 Colony-PCR 34
2.2.8.3 Restriction endonuclease digestions 35
2.2.8.4 Nucleotide sequencing and analysis 35
2.2.9 Plasmid constructs 35
2.2.10 Expression of recombinant proteins in *E. coli* 38
2.2.11 Periplasmic protein extraction 38
2.2.12 Analysis of recombinant proteins 39
2.2.12.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 39
2.2.12.2 Western blot analysis 39
2.2.13 Optimization of recombinant protein expression in *E. coli* 40
2.2.14 Protein concentration determination 41
2.3 RESULTS 41
2.3.1 Construction of recombinant pET-28a(+) bacterial expression vectors 41
2.3.1.1 Construction of expression vectors harbouring different versions of the *etx* gene 42

2.3.1.2 Construction of expression vectors harbouring fusion genes 45
2.3.2 Analysis of recombinant protein expression in *E. coli* BL21-Gold(DE3) 50
2.3.2.1 SDS-PAGE and Western blot analyses 50
2.3.2.2 Cellular localization of the recombinant ETX0 protein 53
2.3.3 Optimization of recombinant protein expression in *E. coli* BL21-Gold(DE3) 54
2.3.3.1 Recombinant protein expression in different cultivation media 56
2.3.3.2 Optimization of the IPTG inducer concentration 56
2.4 DISCUSSION 60

CHAPTER 3 65

Purification of recombinant *Clostridium perfringens* Type D epsilon toxins and a flagellin-toxin fusion protein

3.1 INTRODUCTION 66
3.2 MATERIALS AND METHODS 68
3.2.1 Cell culture 68
3.2.2 Bacterial strains and culture conditions 68
3.2.3 Recombinant protein expression in *E. coli* 69
3.2.4 Small-scale purification of recombinant proteins 69
3.2.5 Ion exchange chromatography 70
3.2.6 Nickel affinity chromatography 70
3.2.6.1 Purification of NC-ETX1 with a Ni-NTA resin 71
3.2.6.2 Purification of ETX1 and NC-ETX1 with a Ni-TED resin 71
3.2.7 Protein concentration determination 72
3.2.8 Activation of the recombinant ETX0 protein 72
3.2.9 Cytotoxicity assays 73
3.2.10 Protein identification by mass spectrometry 73
3.3 RESULTS 74
3.3.1 Purification and characterization of recombinant proteins 76
3.3.2 Purification of recombinant ETX0 by ion exchange chromatography 79
3.3.3 Purification of the recombinant NC-ETX1 and ETX1 proteins 79
3.2.3.1 Purification of recombinant NC-ETX1 with Ni-NTA resin 79
3.2.3.2 Purification of recombinant ETX1 and NC-ETX1 with Ni-TED resin 81

3.3.4 Trypsin treatment of the recombinant ETX0 protein 83
3.3.5 Cytotoxic activity of recombinant proteins 83
3.4 DISCUSSION 84

CHAPTER 4 **91**
CONCLUDING REMARKS

REFERENCES **96**

APPENDICES **121**

- A1: Nucleotide sequence of the native and *E. coli* codon-optimized *etx*0 gene. 122
- A2: Nucleotide sequence of the *etx*1 gene. 123
- A3: Nucleotide sequence of the Δ*hag::etx*1 gene. 124
- A4: Cytotoxicity assays. 126

LIST OF ABBREVIATIONS

Å	Angstrom
ACN	acetonitrile
Amp^r	ampicillin resistance
APC	antigen presenting cells
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
C	carboxy
ca.	approximately
CFA	complete Freund's adjuvant
cm	centimetre
CV	column volume
DC	dendritic cells
DEA	Diethanolamine
DEA	diethanolamine
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
DTT	dithiothreitol
ε	epsilon
e.g.	*exempli gratia* (for example)
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	*et alia* (and others)
ETX	epsilon toxin
FA	formic acid
Fig.	figure
GST	glutathione-S-transferase
h	hour
HPLC	high-performance liquid chromatography
i.e.	*id est* (that is)
IC_{50}	inhibitory concentration 50
IEC	ion exchange chromatography

IFN	interferon
IgG	immunoglobulin G
IkB kinase	Ikappa B kinase
IL	interleukin
IL-1R	interleukin-1 receptor
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
IRAK	IL-1R-associated kinase
Kan^r	kanamycin resistance
kb	kilobase pairs
kDa	kilodalton
kg	kilogram
kV	kilovolt
l	litre
LAT	latex agglutination test
LB	Luria-Bertani
LD_{50}	lethal dose 50
LRR	leucine-rich repeats
M	molar
m/z	mass-to-charge ratio
MAP kinase	mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney cells
mg	milligram
min	minute
ml	millilitre
MLD	minimum lethal doses
mm	millimetre
mM	millimolar
MNT	mouse neutralization test
MS	mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MyD88	myeloid differentiation factor 88
N	amino
NF-kB	nuclear factor kappa B
ng	nanogram
Ni-NTA	nickel-nitrilotriacetic acid
Ni-TED	nickel-tris-carboxymethyl ethylene diamine

nm	nanometre
no.	number
OBP	Onderstepoort Biological Products
OD	optical density
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular patterns
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PMS	phenazine methosulfate
PSB	protein solvent buffer
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Th	T helper cells
TLR	Toll-like receptor
TMB	5,5'-tetramethylbenzidine
TRAF6	Tumor necrosis factor receptor-associated factor 6
Trx	thioredoxin
U	units
UHQ	ultra-high quality
UV	ultraviolet
V	volts
v.	version
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
%	percentage
× *g*	centrifugal force
°C	degrees Celsius
µF	microfarad
µg	microgram
µl	microlitre

µM	micromolar
α	alpha
β	beta
ι	iota
λ	lambda
Ω	ohm

LIST OF FIGURES

Fig. 1.1 Schematic illustration of the primary structure of the *etx* gene product. 11

Fig. 1.2 Structures of members of the aerolysin-like β-pore-forming family, as solved by X-ray crystallography. 12

Fig. 1.3 The innate and adaptive immune systems. 18

Fig. 1.4 Structure of the flagellin protein. 21

Fig. 1.5 A schematic representation of the propelling structure of bacterial flagella. 21

Fig. 2.1 Schematic representation of expression constructs generated during the course of this study. 36

Fig. 2.2 Agarose gel electrophoretic analysis of the recombinant plasmid pEtx0. 44

Fig. 2.3 Agarose gel electrophoretic analysis of recombinant plasmid pEtx1, and the intermediate plasmid pSK-Etx1 used in its construction. 44

Fig. 2.4 Agarose gel electrophoretic analysis of the recombinant plasmid pNCetx1, and the intermediate plasmids pSK-NC and pSK-Letx1 used in its construction. 47

Fig. 2.5 Agarose gel electrophoretic analysis of the recombinant plasmid pHagetx1, and the intermediate plasmids pSK-Hag and pSK-Letx1 used in its construction. 49

Fig. 2.6 SDS-PAGE analysis of recombinant epsilon toxin and flagellin-toxin proteins Expressed in *E. coli* BL21-Gold(DE3). 51

Fig. 2.7 Western blot analysis of recombinant epsilon toxin and flagellin-toxin proteins expressed in *E. coli* BL21-Gold(DE3). 52

Fig. 2.8 Cellular localization of the recombinant ETX0 protein. 55

Fig. 2.9 Recombinant ETX0 protein production in *E. coli* BL21-Gold(DE3) cells using different cultivation media. 57

Fig. 2.10 Recombinant protein production in *E. coli* BL21-Gold(DE3) cells using different concentrations of the inducer. 59

Fig. 3.1 Western blot analysis following expression and purification of the recombinant ETX0, ETX1 and NC-ETX1 proteins. 75

Fig. 3.2 Purification of the recombinant ETX0 protein by ion exchange chromatography. 78

Fig. 3.3 Purification of recombinant NC-ETX1 by Ni-NTA affinity chromatography. 80

Fig. 3.4 Purification of the recombinant ETX1 and NC-ETX1 proteins by Ni-TED affinity chromatography. 82

Fig. 3.5 Western blot analysis of the recombinant ETX0 protein following treatment with trypsin. 85

Fig. 3.6 Sequence alignment of the signal peptide sequence of the epsilon toxin with signal peptides used for secretory production of recombinant proteins in *E. coli*. 87

LIST OF TABLES

Table 1.1	*Clostridium perfringens* toxinotypes and their associated human and animal diseases	5
Table 2.1	Bacterial strains and plasmids used in this study	29
Table 2.2	Primers used in this study	31
Table 3.1	Bacterial strains used in this study	68
Table 3.2	Cytotoxicity of the recombinant ETX0, ETX1 and NC-ETX1 proteins against MDCK cells	85

CHAPTER 1

Literature Review

1.1 GENERAL INTRODUCTION

Livestock production and rearing is an important part of the economy of many countries and is considered to be the greatest contributor to the livelihood of the majority of the poor around the world (Perry *et al.*, 2002). The steady development in animal production and subsequent food security has for centuries been hampered by animal diseases. To date, vaccination continues to be the most effective way of controlling animal diseases, either through preventing mortality or reducing morbidity (Rogan and Babiuk, 2005; Meeusen *et al.*, 2007). Moreover, vaccines also help to ensure a safe and efficient global food supply by reducing the transmission of zoonotic infections from animals to humans and by reducing the need for low-level antibiotics (Roth, 2011). Indeed, vaccines have been the only tools capable of eradicating animal diseases, either globally as exemplified by the eradication of Rinderpest (Roeder *et al.*, 2004) or at a regional level as exemplified by the elimination of foot-and-mouth-disease in some South American countries (Rweyemamu and Astudillo, 2002).

Enterotoxemia, or pulpy kidney disease, is an economically important disease of sheep, goats and calves worldwide. The disease is caused by systemic effects of the epsilon toxin produced by the anaerobic bacterium *Clostridium perfringens* Type D (Niilo, 1980; Songer, 1996; Uzal, 2004). Due to the almost certain death of affected animals, there is no form of treatment of the disease. The only practical means of controlling the occurrence of enterotoxemia is to immunize animals with an epsilon toxoid (Titball, 2009). In South Africa, enterotoxemia in livestock is controlled using a commercially available vaccine produced by Onderstepoort Biological Products (OBP), Pretoria, South Africa. The vaccine is produced by growing a toxigenic strain of *C. perfringens* Type D in enriched broth that results in the production of the epsilon prototoxin, which is clarified and treated with trypsin to convert the prototoxin into active toxin. The epsilon toxins are subsequently treated with formaldehyde to produce innocuous toxoids that are then formulated into a vaccine. Despite the efficacy of this vaccine, it is costly to produce and relies on problematic anaerobic culturing of the vaccine production strain. Moreover, problems commonly experienced in the production of enterotoxemia vaccines are that the epsilon toxin is not produced in high levels in culture and that toxin production by cell culture declines rapidly due to instability of the vaccine production strains (B. Weyers, OBP, personal communication; Goncalves *et al.*, 2009).

In contrast to the above approach, modern vaccines are increasingly based on purified subunit antigens or antigens produced by recombinant DNA technology (Meeusen *et al.*, 2007). Although these approaches have led to improved vaccine safety profiles, the loss of pathogen-derived structures has considerably reduced their immunogenicity *in vivo* (McKee *et al.*, 2007). Consequently, additional substances, referred to as adjuvants, are often included in vaccine formulations in order to enhance the immune response. Based on an increased understanding of the immune system's ability to identify and respond to different pathogens, novel adjuvants have also been identified that can be used to enhance a pathogen-specific immune response and is strong enough to prevent future infection (Mutwiri *et al.*, 2007; Miyaji *et al.*, 2011; Demento *et al.*, 2011). One such novel adjuvant is flagellin, the major structural component of flagella, which has been shown to be a potent activator of a broad range of cell types involved in innate and adaptive immunity (Honko and Mizel, 2005; Huleatt *et al.*, 2007). Notably, the plasticity of flagellin has allowed for the generation of a range of flagellin-antigen fusion proteins that have proven to be effective vaccines in animal models (Huleatt *et al.*, 2008; Song *et al.*, 2008; Delaney *et al.*, 2010).

In this review of the literature, knowledge regarding *C. perfringens* Type D will be presented and aspects relating to the production, structure and mode of action of the epsilon toxin will also be covered. This will be followed by a description of enterotoxemia vaccines, with an emphasis on the use of adjuvants as a means to enhance the immune response. The section will be concluded with a description of the aims of this study.

1.2 ENTEROTOXEMIA IN LIVESTOCK

1.2.1 Introduction to *Clostridium perfringens* Type D

The *Clostridium* genus encompasses more than 80 species that form a diverse group of Gram-positive, rod-shaped bacteria with the ability to form spores. These bacteria are obligate anaerobes, but some species can survive in an oxygen-rich environment for extended periods of time (Lyerly *et al.*, 2004). *Clostridium perfringens* is one of the most pathogenic species in the *Clostridium* genus (Petit *et al.*, 1999; Lyerly *et al.*, 2004). Specifically, *C. perfringens* is an encapsulated, non-motile obligate anaerobic bacterium that forms sub-terminal spores. Colonies of *C. perfringens* grown on blood agar are smooth, round, greyish in colour and are

surrounded by a double zone of hemolysis. *C. perfringens* is oxidase- and catalase-negative, and produces acid and gas from sugars. It is lecithinase- and gelatinase-positive, and indole-negative. Inoculation of the bacterium into litmus milk results in coagulation and the production of gas (Lyerly *et al.*, 2004).

Clostridium perfringens is ubiquitous in the environment, being found in soil, water and sewage. Moreover, they can also be found in the gastrointestinal tract of humans and animals where they form part of the common gastrointestinal flora (Lyerly *et al.*, 2004; Aitken, 2007). Since the discovery of *C. perfringens* as the causative agent of gangrene at the end of the 19th century, the bacterium has been the object of intensive study (Rood, 1998). In addition to causing food poisoning, enteritis, gangrene and puerperal septicemia in humans, various forms of acute enteritis and fatal enterotoxemias in animals have been attributed to *C. perfringens* (Niilo, 1980; Finnie, 2004) (Table 1.1). *C. perfringens* produces the largest number of toxins of any bacteria. As many as 17 exotoxins of *C. perfringens* have been described in the literature, but a definitive role in pathogenesis has been demonstrated for only a few (McDonel, 1980; Sakurai, 1995; Rood, 1998; Garcia *et al.*, 2013) For classification purposes, the species is classified into five toxinotypes (A, B, C, D and E) according to the production of four major toxins, namely alpha (α or CPA), beta (β or CPB), epsilon (ε or ETX) and iota (ι or ITX) (McDonel, 1980; Petit *et al.*, 1999). Type A is defined as strains producing α toxin, Type B as strains producing α, β and ε toxins, Type C as strains producing α and β toxins, Type D as strains producing α and ε toxins, and Type E as strains producing α and ι toxins. Since the respective toxins are antigenic, typing is achieved by neutralization of the toxins with type-specific antisera using mice or guinea pigs as test animals (Brooks *et al.*, 1957; McDonel, 1980). The other dentified toxins of *C. perfringens* are produced in different combinations with perfringolysin O (PFO), enterotoxin (CPE) and beta2 toxin (CPB2) as minor lethal toxins (Uzal *et al.*, 2010).

Although both *C. perfringens* Types B and D produce the epsilon (ε) toxin, they cause different diseases in livestock animals. *C. perfringens* Type B, which also produces β-toxin, is the aetiological agent of dysentery in newborn lambs, whereas *C. perfringens* Type D causes enterotoxemia mainly in sheep and lambs, but also in goats and calves (Niilo, 1980; Songer, 2010). The epsilon toxin is one of the most potent toxins known and its lethal activity ranges just below that of botulinum and tetanus toxins (Cavalcanti *et al.*, 2004). Based on structural data, the epsilon toxin has been grouped as a member of the family of

aerolysin pore-forming toxins (Cole *et al.*, 2004). However, its precise mode of action, accounting for its high potency, remains to be defined (Knapp *et al.*, 2010).

Table 1.1 *Clostridium perfringens* toxinotypes and their associated human and animal diseases (Modified from Cavalcanti *et al.*, 2004)

Toxinotype	Major toxins				Associated diseases	
	CPA	CPB	ETX	ITX	Humans	Animals
A	+	-	-	-	Gangrene, Gastrointestinal diseases	Gangrene Diarrhoea Necrotic enteritis in fowl
B	+	+	+	-	-	Dysentery in new-born lambs Enterotoxemia in sheep
C	+	+	-	-	Necrotic enteritis	Necrotic enteritis in piglets, lambs, calves and foals Enterotoxemia in sheep
D	+	-	+	-	-	Enterotoxemia in lambs, sheep, calves and goats
E	+	-	-	+	-	Enterotoxemia in calves

- No detected toxin production; + Detected toxin production

1.2.2 *C. perfringens* Type D disease

Of the different *C. perfringens* toxinotypes, Type D is widely regarded as the causative organism of fatal enterotoxemia (also referred to as pulpy kidney disease or overeating disease) of sheep, goats and calves found worldwide (Uzal, 2004; Titball, 2009). The most important factor responsible for disease initiation is considered to be disruption of the microbial balance in the gastrointestinal tract of animals. Most cases of Type D enterotoxemia in sheep relate to access to lush pastures after rains in spring and autumn (Bullen, 1963), as well as sudden changes in their diet, usually to feeds rich in highly fermentable carbohydrates (Uzal and Kelly, 1996) or continuous feeding of high levels of feed concentrates (Popoff, 1984). As a consequence, large amounts of undigested carbohydrates enter the small intestine from the rumen and are an excellent substrate for *C. perfringens* growth. The rapid multiplication of Type D bacteria results in the production of large amounts of the epsilon toxin, which, in turn, causes its absorption into the systemic circulation system thereby leading to acute disease and death (Uzal and Kelly, 1996; Uzal *et al.*, 2004).

1.2.3 Clinical symptoms

C. perfringens Type D causes enterotoxemia in sheep of all ages, except newborns (Uzal, 2004). Type D enterotoxemia in lambs is rapidly fatal. Enterotoxemia is the most prevalent in lambs that are 3 to 10 weeks of age and is a predominant cause of death in weaned animals up to 10 months of age, usually those fed rich rations of grain in feedlots (Uzal, 2004; Aitken, 2007). *C. perfringens* Type D causes acute disease in sheep (Lewis, 2000). In the acute form, which is very rapid (few minutes to several hours, no more than 12 hours), the effects of increased epsilon toxin levels on the central nervous system and other tissues cause sudden death, with some animals surviving long enough to display clinical signs such as dullness, retraction of the head, opisthotonus, convulsions, frothing by the mouth and recumbency with paddling immediately before death (Lewis, 2000). Lambs and sheep that died from enterotoxemia show characteristic modifications of the kidneys. Just after death, the kidneys are swollen or congestive, but they autolyse more rapidly than normal, with the cortical parenchyma being totally liquefied (Gardner, 1973a). In addition to the kidneys, the epsilon toxin also accumulates massively in the brain where it produces foci of liquefactive necrosis, perivascular edema and haemorrhage in especially the meninges (Buxton *et al.*, 1976). Focal encephalomalacia occurs sporadically in affected sheep and is characterized by haphazard roaming, blindness, head pressing and an inability to eat (Uzal *et al.*, 1997a).

In goats, *C. perfringens* Type D produces acute, subacute or chronic disease. The acute form occurs more frequently in young animals and is clinically similar to the acute disease in sheep (Blackwell *et al.*, 1991; Blackwell and Butler, 1992; Uzal and Kelly, 1996). The subacute form is more frequently seen in adult goats and is characterized by diarrhea, abdominal discomfort, severe shock, opisthotonos and convulsions. The disease may result in death 2-4 days after onset, but some animals recover (Blackwell *et al.*, 1991). Adult animals can also exhibit chronic disease, which is characterized by agalactia, abdominal discomfort, weakness, anorexia and profuse watery diarrhea that often contains blood and mucus (Blackwell and Butler, 1992). This chronic form may last for days or weeks and may culminate either in death or recovery (Uzal and Kelly, 1996).

In contrast to calves, it appears that Type D enterotoxemia is relatively rare in cattle (Mumford, 1961; Niilo, 1980). More recently, a condition with brain lesions similar to those observed in sheep enterotoxemia has been described in cattle (Fairley, 2005), but a causal

relationship with *C. perfringens* Type D could not be established. Nevertheless, it is possible to produce Type D enterotoxemia in cattle experimentally (Uzal *et al.*, 2002; Filho *et al.*, 2009). The results of these studies have shown distinctive pathological and bacteriological findings that were similar to those observed in sheep.

1.2.4 Pathogenesis

The accumulation of high concentrations of the epsilon toxin in the intestines of livestock animals is thought to result in an increase in the permeability of the intestinal mucosa, thus mediating the passage of toxin into the blood and its subsequent dissemination to the main target organs of the kidneys and brain (Fernandez Miyakawa *et al.*, 2003; Fernandez Miyakawa and Uzal, 2003). Histological analysis of intestinal loops of sheep and goats exposed to the epsilon toxin revealed necrosis of the colonic epithelium in both species, suggesting that alteration of the permeability of the large intestine might play a role in toxin absorption (Fernandez Miyakawa and Uzal, 2003). In mice and rats, high concentrations of epsilon toxin (10^3 MLD/ml) cause an accumulation of fluid in the intestinal lumen, a decrease in transepithelial electrical resistance and an increase in the passage of macromolecules across the intestinal barrier (Goldstein *et al.*, 2009). Moreover, transmission electron microscopy studies revealed that the toxin alters the permeability of the small intestine by opening the mucosa tight junction, indicating that the small intestine might also have a role in toxin absorption (Goldstein *et al.*, 2009). The observation of necrotic cells and gaps in endothelium suggests that the epsilon toxin targets endothelial cells specifically and alters the integrity of the endothelial barrier via the destruction of cells (Adamson *et al.*, 2005; Goldstein *et al.*, 2009). However, the precise mechanism of epsilon toxin-dependent increased permeability of the intestinal barrier remains to be defined.

A characteristic feature of lamb and sheep enterotoxemia is rapid post-mortem autolysis of the kidneys. At 2 to 4 h post-mortem, kidneys show interstitial hemorrhage between tubules and degeneration of the proximal tubule epithelium (Gardner, 1973a). Similar findings have also been observed in mice (Gardner, 1973b). In addition to severe degeneration of the distal tubule epithelium, mice intoxicated with epsilon toxin also show congestion and hemorrhage in the medulla (Soler-Jover *et al.*, 2004). The epsilon toxin binds specifically to the basolateral side of distal tubule epithelial cells, in agreement with the degenerative effects in this epithelium, and also to the luminal surface of proximal tubules, albeit in a non-specific

manner, indicating that the toxin is filtered by the glomerules (Tamai *et al.*, 2003; Soler-Jover *et al.*, 2004). Interestingly, nephrectomy shortens the time to death in mice injected intravenously with epsilon toxin, suggesting that the kidneys play a protective role by trapping the toxin from the circulation system and eliminating it from the animal (Tamai *et al.*, 2003).

In addition to the kidneys, the epsilon toxin also accumulates massively in the brain. This indicates that the toxin passes the blood-brain barrier and recognizes specific cells or sites in the brain. Indeed, epsilon toxin has been shown to alter the integrity of the blood-brain barrier, permitting not only its own passage, but also that of the macromolecule serum albumin (Nagahama and Sakurai, 1992; Finnie and Hajduk, 1992). However, the mechanism of blood-brain barrier perturbation is not yet fully understood. Nevertheless, studies have provided evidence that neurological damage in intoxicated animals is induced by increased vascular permeability in brain blood vessels, leading to vasogenic oedema (Morgan *et al.*, 1975; Finnie and Hajduk, 1992). There is also evidence that the toxin acts directly on neuronal tissues of intoxicated animals. In mice, epsilon toxin causes bilaterally symmetrical lesions in several brain areas including the cerebral cortex, corpus striatum, vestibular area, corpus callosum, lateral ventricles and cerebellum. In contrast, in lambs or sheep, more restricted areas are involved such as basal ganglia, thalamus, subcortical white matter, substantia nigra, hippocampus and cerebellar peduncles (Finnie, 1984; Finnie, 2003). Some neuronal damage also occurs, and consists of swelling, vacuolation and necrosis, mainly in neurons from certain brainstem nuclei, or of cell shrinkage with hyperchromatosis and nuclear pyknosis, most commonly in the cerebral cortex, hippocampus and thalamus (Finnie *et al.*, 1999). In addition to a direct action on neuronal tissues, it has also been suggested that the epsilon toxin exhibits neurotoxicity towards the brain by inducing the release of neurotransmitters. In mice, the lethal activity of the epsilon toxin was reduced by dopamine receptor antagonists and by drugs that directly or indirectly inhibit dopamine release, indicating that the toxin stimulates release of dopamine from dopaminergic nerve endings (Nagahama and Sakurai, 1993). More recently, it was shown that prior injection of either a presynaptic glutamate release inhibitor or a glutamate receptor antagonist protected the rat hippocampus from toxin-induced neuronal damage, indicating that the epsilon toxin also stimulates glutamate release (Miyamoto *et al.*, 2000; Lonchamp *et al.*, 2011).

1.2.5 Diagnosis of enterotoxemia

Large amounts of the epsilon toxin as well as large numbers of *C. perfringens* can usually be observed in the intestinal contents of diseased or dead animals (Uzal and Songer, 2008). Since *C. perfringens* Type D is a natural host of animal intestines, isolation of the bacterium is not by itself diagnostic. Consequently, the diagnosis of enterotoxemia is based on clinical signs and post-mortem findings, but identification of toxins in the intestinal contents is required to confirm the diagnosis.

The most widely used method for toxin detection is the mouse neutralization test (MNT), but this *in vivo* assay has become undesirable because of the expense and the large number of laboratory animals required for the test. Moreover, if diagnosis is based solely on toxicological results, it is potentially possible to misdiagnose Type B (produces α, β and ε toxins) infections as Type D (produces α and ε toxins) if the β toxin is destroyed by the action of intestinal trypsin (Uzal and Songer, 2008). To address these concerns, a number of alternative *in vitro* tests have been developed. DNA-based methods such as nucleic acid hybridization and the polymerase chain reaction (PCR) have been developed for detection of the toxin-encoding genes, and were reported to allow for more accurate and complete determination of *C. perfringens* toxinotypes than does testing in animals (Havard *et al.*, 1992; Daube *et al.*, 1994; Moller and Ahrens, 1996; Kalender *et al.*, 2005).

Most of the *in vitro* methods for detection of *C. perfringens* Type D are, however, based on immunoassays for detection of the epsilon toxin. These include immunodiffusion (Beh and Buttery, 1978), immunoelectrophoresis (Henderson, 1984; Tripathi *et al.*, 1992), the latex agglutination test (LAT) (Martin and Naylor, 1994; Marks *et al.*, 1999) and enzyme-linked immunosorbent assays (ELISA) (Sojka *et al.*, 1989; Wood *et al.*, 1991; Uzal *et al.*, 1997b; Uzal *et al.*, 2003). Among these tests, LAT and ELISA have been used the most frequently for laboratory diagnosis of enterotoxemia, especially in cases of sudden death outbreaks in sheep. These tests are simple, cost-effective and provide quantitative results. Moreover, they can be used for both toxin-typing and for the differential diagnosis of *C. perfringens* Types B, C, D and E enterotoxemias (Naylor *et al.*, 1987; El Idrissi and Ward, 1992; Martin and Naylor, 1994).

1.3 THE EPSILON TOXIN OF *C. perfringens* Type D

1.3.1 Epsilon toxin genetics and production

The gene encoding the epsilon toxin, designated as *etx*, is located on extrachromosomal plasmid DNA in both *C. perfringens* Types B and D (Canard *et al.*, 1992; Hughes *et al.*, 2007). In *C. perfringens* Type B isolates the *etx* gene is located on a single plasmid of 65 kb (Sayeed *et al.*, 2010), whereas in Type D isolates five different plasmids, which range in size from 48 to 110 kb, have been identified that harbour the *etx* gene (Sayeed *et al.*, 2007). In addition to the *etx* gene, the larger plasmids from *C. perfringens* Type D were reported to also carry genes encoding for two other toxins, i.e. *C. perfringens* enterotoxin (CPE) and Beta2 toxin (Sayeed *et al.*, 2007). Interestingly, a *C. perfringens* strain can change from one toxinotype to another through the acquisition of a toxigenic plasmid. For example, Hughes *et al.* (2007) demonstrated conjugative transfer of an *etx* plasmid from a *C. perfringens* Type D isolate to a Type A isolate, essentially converting the Type A isolate to a Type D isolate, both genotypically and phenotypically. Additional evidence for genetic exchange among toxinotypes was provided by the finding that a *tcp* locus, which is required for conjugation, is present in some *etx* plasmids from both *C. perfringens* Types B and D isolates (Hughes *et al.*, 2007). It was thus concluded that the horizontal transfer of toxin plasmids may account for the large genetic diversity observed in *C. perfringens* strains.

The epsilon toxin is synthesized during the exponential growth phase of all *C. perfringens* Type D strains as a single protein containing an N-terminal signal sequence of 32 amino acids that directs the export of the prototoxin from the bacterium (Hunter *et al.*, 1992). The inactive secreted prototoxin is converted to the fully active mature toxin by proteolytic cleavage in the gut lumen, either by digestive proteases of the host, such as trypsin and α-chymotrypsin (Bhown and Habeeb, 1977), or by lambda (λ)-protease, which is produced by *C. perfringens* (Minami *et al.*, 1997). However, differences in the toxicity of the mature epsilon toxin have been noted and are dependent on the protease used for activation of the prototoxin. The λ-protease removes 11 N-terminal and 29 C-terminal residues, whereas trypsin together with α-chymotrypsin cleaves 13 N-terminal residues and the same 29 C-terminal residues. Maximal activation of the epsilon toxin occurs with a combination of trypsin and α-chymotrypsin, producing a mature toxin that is 1000-fold more active than the prototoxin, with an LD_{50} of 70 ng/kg in mice (Gill, 1987). In contrast, if trypsin alone is used for activation, only the C-

terminal 22 residues are removed, thus resulting in a lower toxicity in mice with an LD_{50} of 320 ng/kg (Minami *et al.*, 1997). If *C. perfringens* λ-protease is used for activation, the C-terminus is cleaved at the same position as α-chymotrypsin but three extra residues are present in at the N-terminus. In this case, the processed epsilon toxin displays an activity closer to maximal with an LD_{50} of 110 ng/kg (Minami *et al.*, 1997).

Fig. 1.1 Schematic illustration of the primary structure of the *etx* gene product. The epsilon toxin is secreted as an inactive prototoxin, which is post-translationally activated by removal of N- and C-terminal peptides by trypsin (red), chymotrypsin (black), or *C. perfringens* λ-protease (blue) at the indicated postions (Adapted from Bokori-Brown *et al.*, 2011).

1.3.2 Structure of the epsilon toxin

The three-dimensional structure of the epsilon toxin has been determined (Cole *et al.*, 2004). The crystal structure revealed that the toxin is an elongated molecule (100Å × 20Å × 20Å) and contains three domains that are composed mainly of β sheets (Cole *et al.*, 2004) (Fig. 1.2). Domain I of the epsilon toxin consists of a large α helix, followed by a loop and three short α helices. It has been suggested that a cluster of aromatic residues (Tyr_{49}, Tyr_{43}, Tyr_{42}, Tyr_{209} and Phe_{212}) present in this domain could be involved in cell receptor binding (Cole *et al.*, 2004). Domain II is a β-sandwich composed of a two-stranded sheet with an amphipathic sequence (His_{151}-Ala_{181}), which contains alternate hydrophobic-hydrophilic residues characteristic of membrane-spanning β-hairpins (Cole *et al.*, 2004; Knapp *et al.*, 2009). Domain II has therefore been predicted to be a channel-forming domain. Supporting evidence for its importance in pore formation have been provided by Pelish and McClain (2009) who showed that mutagenesis of this domain prevented pore formation, but not receptor binding. Domain III is also a β-sandwich and contains one four-stranded sheet and one three-stranded sheet. Domain III is proposed to be involved in the monomer-monomer interaction required for oligomerization of the epsilon toxin after the C-terminal amino acid residues have been removed by protease cleavage (Miyata *et al.*, 2001; Cole *et al.*, 2004).

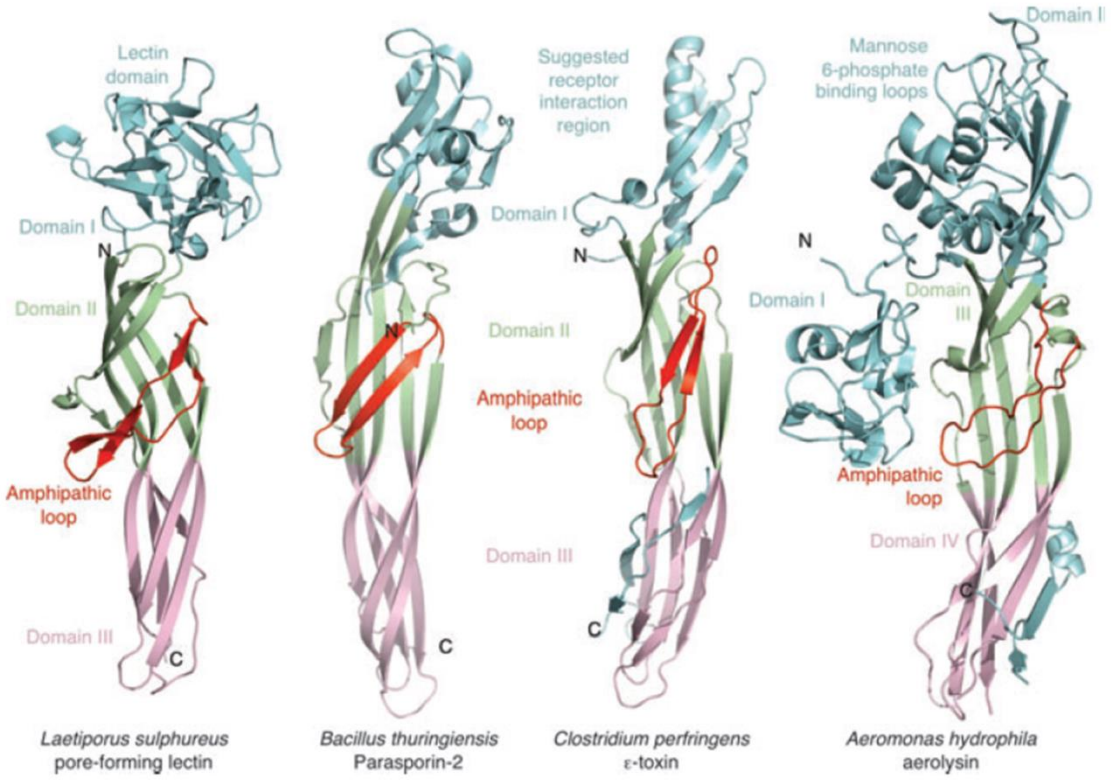


Fig. 1.2 Structures of members of the aerolysin-like β-pore-forming family, as solved by X-ray crystallography. Coloured cyan indicates the N-terminal membrane-interacting and other non-related regions, pale green and pink for domains important for oligomerization and membrane interaction, and red for the β-hairpin predicted to insert into the membrane (Adapted from Bokori-Brown *et al.*, 2011).

It has been noted that the overall fold of the epsilon toxin structure shows similarity to aerolysin from the Gram-negative bacterium *Aeromonas hydrophila*, to parasporin-2 (PS-2) from *Bacillus thuringiensis*, and to a pore-forming lectin, LSL, from *Laetiporus sulphureus* (Knapp *et al.*, 2010). Despite the low amino acid sequence identity (< 20%) between the above proteins, their structures show similar β-sheet arrangements (Fig. 1.2) in their two C-terminal domains (Domains III and IV in aerolysin, and Domains II and III in the others). In addition to the structural similarity, the epsilon toxin also shares other properties with aerolysin and PS-2. Not only are they all secreted as prototoxins and activated by proteolytic cleavage of N- and C-terminal sequences, but they are also all capable of forming pores (Knapp *et al.*, 2010). In this regard, it is interesting to note that despite evidence for the epsilon toxin being capable of forming pores, the exact mechanism through which this occurs is not yet known. Nevertheless, a mechanism of pore formation has recently been proposed

by Bokori-Brown *et al.* (2011). According to this prediction, the prototoxin is secreted by the bacterium and activated by *C. perfringens* λ-protease or by host proteases such as trypsin and/or α-chymotrypsin. Following receptor binding, the activated epsilon toxin oligomerizes on the cell surface, which may lead to formation of a pre-pore complex. The final step of pore formation might involve unfolding of the amphipathic hairpin and its insertion into the cell membrane to form the walls of the pore.

1.4 PREVENTION OF ENTEROTOXEMIA DISEASE

Since the epsilon toxin is highly potent and fast acting, causing death in animals within minutes to hours after intoxication, it may not be possible to treat *C. perfringens* Type D enterotoxemia. Consequently, control and prophylaxis of the disease is based on systematic vaccination of herds (Uzal *et al.*, 2004; Titball, 2009).

1.4.1 Epsilon toxoided vaccines

Currently, epsilon toxoided vaccines are used to control enterotoxemia in sheep and goats. Toxoid vaccines are made by treating a toxin with heat or chemicals to destroy its toxicity, whilst still retaining its antigenicity. Indeed, these vaccines have been used extensively over the past decades to prevent disease in domesticated livestock (Titball, 2009). Classically, toxin-based vaccines are prepared by treating *C. perfringens* Type D culture filtrates with formaldehyde to inactivate the components (Habeeb, 1969). Since relatively crude culture filtrates are used, the vaccines are likely to contain additional proteins to the epsilon toxoid. Moreover, localization of the *etx* gene on the extrachromosomal plasmids can result in loss of toxigenicity after successive passages of the bacterial culture during vaccine production (Goncalves *et al.*, 2009). Although it is not known which type of immunity is induced by these toxoid vaccines, it is certain that they do not confer long-lasting immunity. Typical immunization regimens involve vaccination of lambs at the age of 3 months for the first time and then with an alum-based vaccine 4 to 6 weeks later. Sheep are boosted every 12 months, whereas goats are boosted every 3-4 months (Uzal *et al.*, 1998). Although the toxoid vaccines are effective in preventing enterotoxemia in goats and sheep, there are reports of variable immune responses following vaccination (Uzal *et al.*, 1998). Moreover, inflammatory responses following vaccination have been reported to lead to reduced feed consumption (Stokka *et al.*, 1994).

1.4.2 Recombinant epsilon vaccines

As a consequence of bacterial cultivation, varying amounts of the epsilon toxin is produced and secreted. Consequently, alternative approaches have been investigated whereby greater amounts of the epsilon toxin can be produced reliably. These approaches involve using genetic engineering to produce the toxin and then using the recombinant protein for toxoiding (Goswami *et al.*, 1996; Chandran *et al.*, 2010; Lobato *et al.*, 2010; Souza *et al.*, 2010). The results of these studies have shown that the derived recombinant epsilon toxoid is immunogenic in rabbits, and immunization of sheep, goats and cattle with the recombinant epsilon toxoid was reported to produce higher anti-epsilon antibodies titres compared to the level considered to be protective against enterotoxemia (Lobato *et al.*, 2010). However, a more promising approach may entail the production of recombinant genetically detoxified epsilon toxin mutants that are biologically inactive but still retain the toxin immunogenicity. In this regard, Oyston *et al.* (1998) reported the production of site-directed mutants of the epsilon toxin of which one of the variant toxins was shown to be non-toxic to mice. Following immunization of mice with the variant toxin, it was subsequently shown that the mice were protected against a challenge with 1000 MLD doses of the wild-type epsilon toxin.

In addition to inactivated and recombinant vaccines, there has also been a growing interest in the use of anti-epsilon toxin polyclonal and monoclonal antibodies as a means to prevent the toxic effects of the toxin. The passive transfer of polyclonal antibodies against the toxin into newborn lambs has been achieved by injection (Odendaal *et al.*, 1989) and by feeding the animals colostrum that contained antibodies (Clarkson *et al.*, 1985). More recent reports have described the generation of monoclonal antibodies that are able to protect cultured cells (Percival *et al.*, 1990; McClain and Cover, 2007) and mice (Percival *et al.*, 1990) from intoxication. The finding that a single monoclonal antibody is able to provide good protection indicates that a single epitope is required for the induction of protection. McClain and Cover (2007) mapped the location of the epitope recognized by the protective monoclonal antibody to amino acids 134-145 (peptide sequence: SFANTNTNTNSK), which overlaps the putative membrane inserting loop of the epsilon toxin. Although these antibodies may find application in the prevention or treatment of enterotoxemia, note should be taken that the rapidity of onset of symptoms in animals would make treatment of epsilon intoxication difficult.

1.5 VACCINE ADJUVANTS

A vaccine should ideally protect by generating a quick, solid and durable immunity without causing disease or serious side-effects (Meeusen *et al.*, 2007; Heegaard *et al.*, 2011). Thus, vaccines aim at prophylactically inducing effector molecules and cells that are capable of eliminating a pathogen as quickly as possible. For this elimination to be successful, a range of host immune responses is required (Shijns, 2001; Mutwiri *et al.*, 2007; Akira, 2011). One major disadvantage of inactivated and recombinant subunit vaccines is the fact that they do not replicate in the host and, as such, have a limited ability to mimic the immune response of the pathogen they are expected to generate protection against. It is therefore important that vaccine formulations be designed in such a way that suitable immune responses are generated.

In 1925, Ramon demonstrated that it was possible to artificially augment antigen-specific antibodies to diphtheria and tetanus toxoids when substances such as agar, metallic salts, lecithin and saponin were administered prior to vaccination (Aucouturier *et al.*, 2001; Petrovsky and Aguilar, 2004). The augmenting substance, called adjuvant (from the Latin "*adjuvare*" meaning to help), has since been shown to be capable of significantly increasing the levels of protection afforded by a vaccine. An ideal adjuvant has been defined as a substance that should not elicit an immune response against itself, it should promote both cell-mediated and humoral immunity, and it must be easy to use, stable and convenient to inject (Aguilar and Rodríguez, 2007). In the case of veterinary vaccines, an adjuvant should furthermore display minimal side-effects that may negatively affect the growth of animals, production rate, and the comfort and welfare of animals (Heegaard *et al.*, 2011).

To date, a large variety of adjuvants have been developed. Based on their origin, adjuvants can be classified into plant or vegetable products (e.g. saponins and vegetable oils), bacterial products (e.g. cholera toxin, lipopolysaccharides mainly from Gram-negative bacteria and muramyl dipeptide from mycobacteria that is used in complete Freund's adjuvant), chemical compounds (e.g. aluminum-based adjuvants, surfactants, emulsions, nanoparticles and polymeric microspheres) and cytokines (e.g. IL-2 and IFN-γ) (Audibert and Lise, 1993; Cox and Coulter, 1997; Singh and O'Hagan, 2003). Although little is known regarding their mechanism of action that contributes to vaccination-induced immunity, vaccine adjuvants nevertheless display a number of beneficial effects. In addition to increasing the potency of

antigenically weak peptides, they may also enhance the speed, vigour and persistence of the immune response to antigens. Moreover, vaccine adjuvants can allow for a decrease in the amount of antigen required and therefore reduces the cost of the vaccine (Coffman *et al.*, 2010).

In the following sections, different immune responses as well as adjuvants used in commercial epsilon toxoided vaccines will be highlighted. Particular attention will be paid to the use of flagellin as a potentially novel adjuvant for use in enterotoxemia vaccines.

1.5.1 Innate and adaptive immunity

Immune responses are typically categorized into either innate immunity or adaptive immunity, which is further divided into humoral immunity and cellular immunity (Akira, 2011). Humoral immunity is involved in the eradication of extracellular pathogens by generating neutralizing antibodies, which are produced by B cells, via a Th2 type humoral response (Schijns, 2001; Akira, 2011). In contrast, cellular immunity is responsible for the eradication of intracellular pathogens and is mediated by killer T cells through a Th1 type humoral response (Schijns, 2001; Akira, 2011). Both the T and B cells express unique receptors on their cell surface and are therefore capable of recognizing a vast number of different antigens. Upon infection with a pathogen, the T and B cells with the corresponding receptors are activated, and results in the induction of killer T cell development and antibody production (Fig. 1.3). Concomitantly, memory T and B cells are generated that allows the host to respond more rapidly when infection with the same pathogen occurs (Schijns, 2001; Akira, 2011).

The innate immunity is mediated by leukocytes, macrophages and dendritic cells, which are collectively called phagocytes because they engulf and kill microbes (Beutler, 2004; Akira, 2011). In the case of dendritic cells, they have an additional role of presenting antigenic peptides derived from microbes to T cells (Fig. 1.3). In contrast to the adaptive immune system, the innate immune system does not confer long-lasting immunity on the host. Consequently, the innate immune system is thought to provide an immediate defense against infection and it has been considered to represent a temporary system until adaptive immune responses can be triggered. Indeed, activation of innate immunity appears to be a prerequisite for the induction of the adaptive immune system (Akira, 2011). The innate immune system is

highly developed in its ability to discriminate self from foreign pathogens. In this regard, antigen-presenting cells (APCs), which are activated dendritic cells, play a crucial role in pathogen recognition and trigger different immune effectors according to the particular microbial type (Akira *et al.*, 2006). The APCs are activated by innate receptors on their membrane that recognize highly conserved pathogen-associated molecular patterns (PAMP) expressed by pathogens. These PAMPs therefore provide the APCs with specific signals that guide the subsequent immune response (Akira *et al.*, 2006). The innate pathogen-specific receptors comprise a family of evolutionarily conserved receptors, designated Toll-like receptors (TLRs). The TLRs are type 1 integral membrane glycoproteins that are characterized by extracellular domains containing varying numbers of leucine-rich repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin-1 receptor (IL-1R), termed the Tol1/IL-1R homolog (TIR) domain (Bowie and O'Neill, 2000). Based on their primary sequences, TLRs can be divided into several subfamilies, each of which recognizes related microbial components. TLR1, TLR2 and TLR6 recognize lipids, whereas TLR7, TLR8 and TLR9 recognize nucleic acids, and TLR5 recognizes flagellin. In contrast, TLR4 recognizes a diverse collection of ligands such as lipopolysaccharides, fibronectin and heat-shock proteins (Akira *et al.*, 2006; Kawai and Akira, 2010).

Based on the above, it follows that the role of vaccines is primarily to generate memory cells against specific pathogens that then allows for a more rapid and vigorous response in case of repeated infection with the same pathogen. Since innate immune cells play an important role in stimulating the adaptive immune response, it furthermore follows that the inclusion of adjuvants in the vaccine formulation that stimulate innate immune cells may better tailor the adaptive immune response so that the expected protective immune response can be induced.

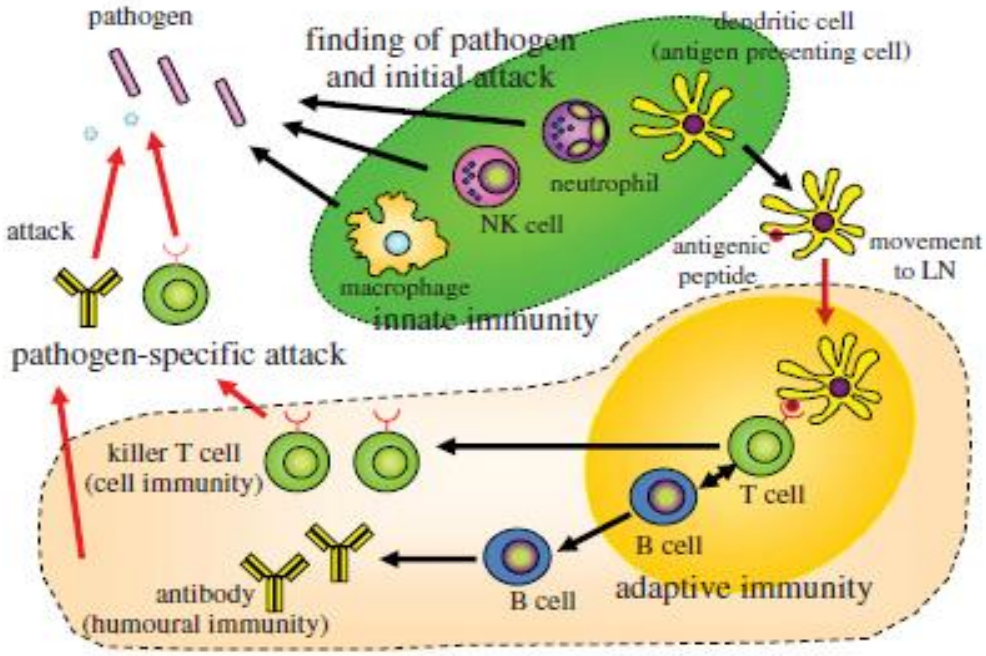


Fig. 1.3 The innate and adaptive immune systems. Following infection with a pathogen, the innate immune system is activated first and participates in the initial attack against the pathogen. Among the cells involved in innate immunity, dendritic cells act as antigen-presenting cells (APCs) and migrate from the infected tissue to the regional lymph nodes where they present the antigen to T cells. The adaptive immune system is subsequently activated and the production of antibodies and killer T cells are induced, which specifically attack the pathogen. Abbreviations: NK - natural killer cells; LN -lymph node (Adapted from Akira, 2011).

1.5.2 Adjuvants used in epsilon toxoided vaccines

Although aluminum salts are the most commonly used adjuvants in the formulation of vaccines against *C. perfringens* Type D enterotoxemia, liposomes have also been investigated experimentally as an alternative adjuvant for use in these toxoided vaccines (Uzal *et al.*, 1999; Titball, 2009). Liposomes are spherical vesicles formed by a hydrated phospholipid bilayer that surrounds an aqueous core (antigen) and act as both a vaccine delivery agent and adjuvant. Liposomes are mainly composed of amphiphillic lipids such as phosphatidylcholine and cholesterol (Demento *et al.*, 2011). The efficacy of liposomes as adjuvants is dependent on the number of lipid layers, electric charge, as well as their composition and method of preparation (Gregoriadis *et al.*, 1987). Although a liposome adjuvant has been evaluated for its ability to enhance the efficacy of epsilon toxoided vaccines, it was reported that no significant antibody responses could be detected (Uzal *et al.*, 1999). Consequently, liposomes are not used in commercially available epsilon toxoided vaccines.

Aluminum salts have been used as vaccine adjuvants since their discovery more than 80 years ago, based on the empirical observation that their inclusion in vaccine formulation enhanced the immune response to antigens (Dementо *et al.*, 2011). Aluminum salts are generally insoluble, gel-like precipitates of aluminum hydroxide [$Al_2(OH)_3$], aluminum phosphate ($AlPO_4$) or alum [potassium aluminum sulfate, $KAl(SO_4)_2.12H_2O$]. With aluminum hydroxide and aluminum phosphate, the antigen is adsorbed onto pre-formed aluminum gels, whereas alum is used to precipitate the antigen in the presence of phosphate, sulphate and bicarbonate anions. The latter results in vaccines that comprise a mixture of aluminum phosphate and aluminum hydroxide (Brewer, 2006). Their mechanism of action is not fully understood, but aluminum salts are believed to act as a depot for vaccine antigen components that enhances antigen uptake by antigen presenting cells (APCs) (Bowersock *et al.*, 1999; Brewer, 2006). Due to its particulate nature, the antigens absorbed by the aluminum salt are likely also presented in a particulate multivalent form that is thought to be more efficiently internalized by APCs (Spickler and Roth, 2003; Morefield *et al.*, 2005). Vaccines formulated with aluminum salts predominantly stimulate the Th2-like humoral immune response and have little or no effect on Th1-type responses, which are instrumental for protection against many pathogens (Brewer, 2006; Miyaji *et al.*, 2011).

1.5.3 Flagellin adjuvants

Flagella, which are composed of a basal body, hook, motor and filament, are bacterial cell-surface appendages that play a major role in motility and chemotaxis (Berg, 2003). The filament comprises flagellin monomers and analysis of the amino acid sequences of these monomers from both Gram-negative and Gram-positive bacteria revealed the presence of highly conserved sequences at the N and C termini, whereas the region between the conserved termini exhibits substantial variability in length and sequence and has therefore been termed the hypervariable region (Joys, 1985; Beatson *et al.*, 2006). The crystal structure of a *Salmonella* flagellin has been solved (Yonekura *et al.*, 2003), revealing it to be a boomerang-shaped protein with four major domains (Fig. 1.4). The α-helical N and C termini (ca. 50 amino acid residues each) comprise domain D0 and are responsible for polymerization of the protein. The second domain, D1, is also primarily α-helical but contains highly conserved regions involved in flagellin signaling. In contrast, domains D2 and D3 consist mostly of β-strands and span the hypervariable region of flagellin (Yonekura *et al.*, 2003).

In addition to being the building block of the flagellar filament, flagellin has been reported to be a potent inducer of innate immunity (Honko and Mizel, 2005). As indicated previously, microorganisms that invade the host are initially recognized by the innate immune system through different Toll-like receptors (TLRs) (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2010). Of these, TLR5, which is expressed by epithelial cells, monocytes and immature dendritic cells (Akira *et al.*, 2006), is responsible for the detection of flagellin and specifically recognizes the D1 domain that is conserved among different bacterial species (Smith *et al.*, 2003; Mizel *et al.*, 2003) (Fig. 1.5). Binding of flagellin by TLR5 initiates a signaling cascade mediated by adaptor molecules and downstream factors that leads to the induction of genes involved in antimicrobial host defense. Both IL-1R-associated kinases (IRAK) and MyD88 are crucial for flagellin signaling via TLR5 (Moors *et al.*, 2001; Hayashi *et al.*, 2001). MyD88 is an adapter molecule that associates with TLR5 and it contains death domains that are responsible for facilitating the interaction of MyD88 with the IRAK kinases (Medzhitov *et al.*, 1998). Upon stimulation, MyD88 associates with the cytoplasmic portion of the receptor and then recruits IRAK-4 and IRAK-1. After IRAK-1 associates with MyD88, it is phosphorylated by the activated IRAK-4 and subsequently associates with and phosphorylates TRAF6. Phosphorylation of this signal transduction protein leads to the activation of MAP kinases and IkB kinase, which results in activation of NF-kB and subsequent expression of genes involved in inflammatory responses (Akira *et al.*, 2006; Vijay-Kumar *et al.*, 2008).

In addition to the induction of pro-inflammatory and co-stimulatory genes, flagellin also promotes several related innate-immune processes that are essential for the development of an adaptive immune response. These processes include a marked increase in T and B lymphocyte recruitment to secondary lymphoid sites, thereby increasing the likelihood of antigen-specific lymphocytes encountering their cognate antigen (Bates *et al.*, 2008; Mizel and Bates, 2010). Moreover, flagellin not only directly stimulates T cells such as $CD4^+$ and $CD8^+$ to proliferate and produce cytokines (Caron *et al.*, 2005; Braga *et al.*, 2010), but it also induces dendritic cell (DC) maturation (Means *et al.*, 2003; Vincente-Suarez *et al.*, 2009). This is especially important since the DCs play an essential role in the induction of adaptive immune responses, acting as a connection between antigen exposure and activation of naïve T cells in secondary lymphoid sites (Akira, 2011). Thus, the ability to promote DC maturation may be a critical means by which the innate immune response drives a subsequent adaptive response against a pathogen. Indeed, the stimulatory effect of flagellin on DC and T cells is

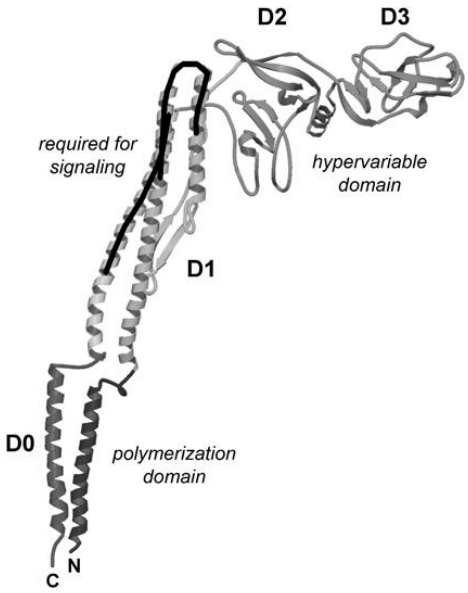


Fig. 1.4 Structure of the flagellin protein. The backbone of flagellin with the four major domains is indicated. (Adapted from Yonekura *et al.*, 2003).

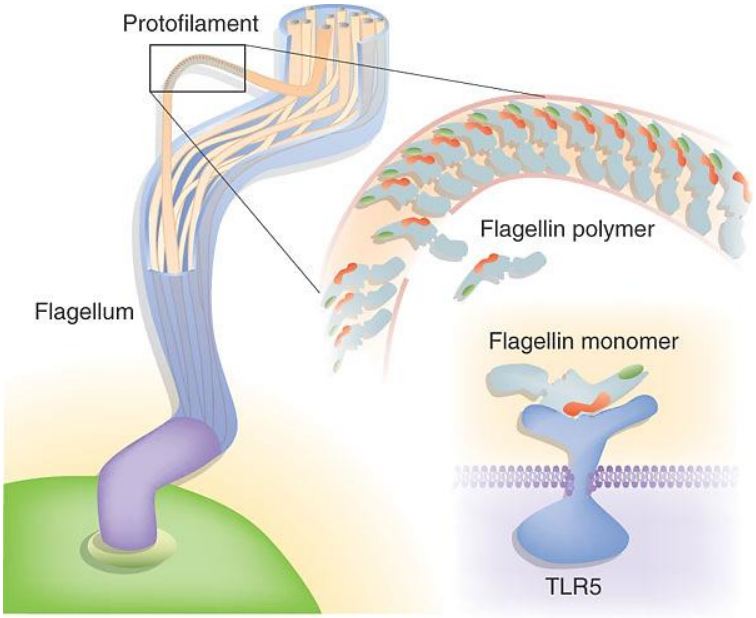


Fig. 1.5 A schematic representation of the propelling structure of bacterial flagella. The flagellin monomers stack together to form flagellin polymers that makes a protofilament of flagellum. The conserved regions of D0 (green) and D1 (red) of the flagellin monomer are shown and the region where the flagellin interacts with TLR5. (Adapted from Reichart *et al.*, 2003).

reported to promote a dramatic increase in T cell-dependent antibody production (Cuadros *et al.*, 2004; Honko *et al.*, 2006; Huleatt *et al.*, 2007). Furthermore, the cumulative effect of the activation of these cell types also results in enhanced activation of B cells and the generation of plasma and memory cells (Mizel and Bates, 2010).

It is important to note that, in contrast to many other TLR agonists and vaccine adjuvants, flagellin tends to produce mixed Th1 and Th2 humoral immune responses (McSorley *et al.*, 2002; Cuadros *et al.*, 2004). It is this feature of flagellin that has made it an attractive adjuvant in vaccine formulation. Although the flagellin and antigen have been used separately (Lee *et al.*, 2006), a markedly enhanced (ca. 10-fold) immune response to flagellin fusion proteins compared with flagellin and antigen have been noted (McSorley *et al.*, 2002; Means *et al.*, 2003). Consequently, in most cases, flagellin fusion proteins are used in which the antigen sequences have been inserted at the N or C terminus or within the hypervariable region of the flagellin protein. It has been suggested that the increased efficacy of flagellin fusion proteins may be due to the more efficient delivery of both the adjuvant and antigen to the same TLR5 on dendritic cells, thereby providing a means for efficient internalization and access to the antigen-processing pathway (Means *et al.*, 2003).

A number of publications have described the adjuvant property of flagellin in the context of a broad range of recombinant vaccines against different viral and bacterial pathogens. For example, recombinant *Salmonella muenchen* flagellin expressing different human influenza virus hemagglutinin epitopes was reported to induce production of protective antibodies and provided long-term immunity in mice (Levi and Arnon, 1996; Jeon *et al.*, 2002). Similarly, recombinant *S. typhimurium* flagellin expressing either the M2e ectodomain of the matrix protein of human influenza virus (Huleatt *et al.*, 2007), the hemagglutinin globular head domain of avian influenza virus (Song *et al.*, 2008) or the envelope protein of West Nile virus (McDonald *et al.*, 2007) generated protective antibodies in mice and provided protection against *in vivo* challenge. More recently, Delaney *et al.* (2010) reported that a recombinant *S. enterica* serovar Enteritidis flagellin expressing the B5R protein of poxvirus not only elicited protection against repiratory challenge with vaccinia virus in mice, but also that the fusion protein was more immunogenic compared to the antigen only. In the case of bacterial pathogens, the insertion of the F1 and V antigens of *Yersinia pestis* in the hypervariable region of *S. enterica* serovar Enteritidis flagellin was reported to produce a highly effective vaccine against respiratory challenge with *Y. pestis* in mice (Honko *et al.*, 2006; Mizel *et al.*,

2009). Furthermore, mice immunized with fusion proteins comprising of *Pseudomonas aeruginosa* type A and B flagellins and both OprF epitope 8 and OprI of *P. aeruginosa* were reported to promote rapid clearance of the bacteria in challenged mice, and the mice had significantly less inflammation and lung damage throughout the infection (Weimer *et al.*, 2009).

1.6 AIMS OF THE STUDY

Enterotoxemia caused by *Clostridium perfringens* Type D is a fatal disease of sheep, goats and calves (Niilo, 1980; Uzal, 2004). The major pathological agent is the epsilon toxin (Songer, 1996), which is produced as an inactive prototoxin and activated by proteolytic enzymes (Bhown and Habeeb, 1977; Minami *et al.*, 1997). Since enterotoxemia in young animals often evolves to a superacute stage, the disease is difficult to treat. Economic losses may, however, be prevented by immunization through vaccination (Titball, 2009). The currently available commercial vaccine relies on problematic anaerobic cultivation of the bacterium, followed by inactivation of the clarified toxin by treatment with formaldehyde. Apart from the fastidious growth requirements of this organism, it has been reported that the production of toxin by these cells declines rapidly and eventually ceases following laboratory passaging of the bacterial cultures. It can be envisaged that the development of a recombinant subunit vaccine against enterotoxemia may alleviate some of the problems associated with the current epsilon toxoided vaccine. However, purified antigens or recombinant subunit vaccines are often poorly immunogenic and require the use of adjuvants in the vaccine formulation to help stimulate protective immunity. In addition to enhancing the uptake of antigens by antigen presenting cells and allowing for progressive release of antigens, adjuvants may also increase the ability of antigens to activate signaling pathways controlling the induction of innate and adaptive immunity (Coffman *et al.*, 2010; Akira, 2011).

An attractive adjuvant for use in vaccines is flagellin, the structural component of flagella. Flagellin is a potent immune activator, stimulating signalling pathways that mediate innate inflammatory responses as well as the development of adaptive immunity (Honko and Mizel, 2005; Mizel and Bates, 2010). Indeed, several studies have indicated that heterologous peptide sequences could be inserted into flagellin to create vaccines that were effective at eliciting humoral immunity in the absence of other adjuvants. Fusion proteins comprising of

flagellins of Gram-negative bacteria, including *Salmonella* spp. (Jeon *et al.*, 2002; Huleatt *et al.*, 2007; Delaney *et al.*, 2010), *Vibrio* spp. (Lee *et al.*, 2006; Liang *et al.*, 2012), *P. aeruginosa* (Weimer *et al.*, 2009) and *E. coli* (Karam *et al.*, 2013), have been used with success as vaccines. To date, the adjuvant properties of flagellins of Gram-positive bacterial species have not been investigated in vaccine formulations. To redress this imbalance, this investigation focused on the flagellin of the Gram-positive bacterium *Bacillus halodurans* strain Alk36. Previous studies have reported on the development of this bacterium for surface display of chimeric flagellin fusions (Crampton *et al.*, 2007; Berger *et al.*, 2011). In these studies, peptides encoding a polyhistidine peptide and the HIV subtype C gp120 epitope were incorporated in the variable region of the flagellin protein, which is encoded by the *hag* gene. Not only were the peptides shown to be successfully displayed on the cell surface, but they were also functional (Crampton *et al.*, 2007). These results therefore provided the impetus for this study, which is aimed at generating a novel vaccine candidate against *C. perfringens* Type D enterotoxemia by making use of flagellin fusion vaccine technology. Consequently, the aims of this study were:

- To construct recombinant expression vectors harbouring full-length or truncated *etx* genes and fusion genes comprising the *Bacillus halodurans* Alk36 flagellin (*hag*) gene and truncated *etx* gene.
- To express the recombinant proteins in *Escherichia coli*.
- To purify the recombinant proteins utilizing affinity and ion exchange chromatography.
- To evaluate the cytotoxicity of the purified recombinant proteins in a mammalian cell culture.

CHAPTER 2

Cloning and expression of *Clostridium perfringens* Type D epsilon toxins and flagellin-toxin fusion proteins in *Escherichia coli*

2.1 INTRODUCTION

Clostridium perfringens is a widespread Gram-positive obligate anaerobic bacterium and is the causative agent of a variety of diseases. It has been associated with gas gangrene and food poisoning in humans, and with a range of severe enterotoxemic diseases in domestic animals (Songer, 1996). *C. perfringens* is classified into five types (from A to E), based on the production of one or more of the four major toxins (alpha, beta, epsilon and iota) (McDonel, 1980; Petit *et al.*, 1999). Epsilon toxin (ETX), produced by *C. perfringens* Type D, is responsible for a rapidly fatal enterotoxemia in economically important livestock such as sheep, goats and calves (Niilo, 1980; Uzal, 2004). Clinical disease occurs when the microbial balance of the gastrointestinal flora is disrupted, often as a consequence of changes in diet, resulting in large amounts of the epsilon toxin being produced (Bullen, 1963; Uzal and Kelly, 1996). The toxin gains access to the systemic circulation and is known to cross the blood-brain barrier (Finnie and Hajduk, 1992), increase vascular permeability in the brain and intestine (Finnie, 2004; Goldstein *et al.*, 2009), and cause kidney damage (Gardner, 1973a; Soler-Jover *et al.*, 2004). Due to the rapid progression of the disease in livestock animals, treatment is generally not possible or practical and the emphasis is placed on prevention by vaccination (Titball, 2009).

The enterotoxemia vaccines currently available are formaldehyde-inactivated whole-cell cultures and culture filtrates of toxigenic *C. perfringens* Type D strains (Titball, 2009). The production of these vaccines suffer from production constraints such as the requirement for strict anaerobic culturing conditions, instability of the vaccine production strains and variable levels of toxin production (Goncalves *et al.*, 2009). A next generation technology for the rapid production of safe and efficacious enterotoxemia vaccines is therefore needed. A promising strategy is the production of recombinant epsilon toxins, since it may allow for greater amounts of protein to be produced with satisfactory qualitative maintenance of the toxoid. Indeed, the full-length epsilon toxin (Goswami *et al.*, 1996; Chandran *et al.*, 2010), as well as N-terminal truncated (Lobato *et al.*, 2010; Souza *et al.*, 2010) and site-directed mutant (Oyston *et al.*, 1998) versions of the toxin have been expressed successfully in *Escherichia coli*. These studies all indicated that the recombinant proteins induced high antibody levels in immunized animals, and protection against lethal challenge has been demonstrated in mice (Oyston *et al.*, 1998) and sheep (Chandran *et al.*, 2010). The results of these studies therefore

indicate that *E. coli* expression of the epsilon toxin can contribute to alleviating the current vaccine production constraints.

In addition to improvements in vaccine production, the current commercial and recombinant enterotoxemia vaccines may also benefit from enhancement of their immunopotency. The epsilon antigens described above have been used in an emulsion with either aluminum hydroxide or complete Freund's adjuvant (CFA). Immunization with protein antigens in CFA results in a strong Th1 cell response, whereas aluminum salts adjuvants induce a strongly polarized Th2 cell response (Shams, 2005; Coffman *et al.*, 2010). However, recent advances in the field of innate immunity have led to the identification and characterization of immunostimulatory molecules that can enhance immune responses (Coffman *et al.*, 2010; Akira, 2011). One such molecule is flagellin, the major structural protein of the flagellar filament of bacteria. Flagellin is a highly effective adjuvant that can induce or boost adaptive immune responses (Honko and Mizel, 2005; Mizel and Bates, 2010). These immune-modulating effects of flagellin are mediated by the activation of antigen-presenting cells (APCs) through binding to Toll-like receptor 5 (TLR5), its cognate receptor (Hayashi *et al.*, 2001). Binding of flagellin to TLR5 triggers innate immune responses and also facilitates the development of adaptive immunity through various mechanisms, including activation and maturation of dendritic cells and expression of cytokines and other co-stimulatory proteins (Akira *et al.*, 2001; Iwasaki and Medzhitov, 2004; Mizel and Bates, 2010). Several studies have shown that genetically fusing an antigen of interest to flagellin not only significantly increases the immunopotency and protective capacity of the antigen, but also generates a significantly more potent vaccine than simple mixing of the antigen and flagellin (Means *et al.*, 2003; Cuadros *et al.*, 2004; Huleatt *et al.*, 2007). Consequently, recombinant flagellin-antigen fusions have been used with success as experimental vaccines against pathogens such as *Yersinia pestis* (Honko *et al.*, 2006; Mizel *et al.*, 2009), *Clostridium difficile* (Ghose *et al.*, 2013), *Pseudomonas aeruginosa* (Weimer *et al.*, 2009), influenza virus (Song *et al.*, 2008; Skountzou *et al.*, 2010) and West Nile virus (McDonald *et al.*, 2007).

In the majority of studies reporting on the usefulness of flagellin-antigen fusion proteins as potential vaccine candidates, the flagellin protein of different *Salmonella* spp. has been used. To date, the immunostimulatory effect of flagellin proteins from Gram-positive bacteria have not been determined. Moreover, it is interesting to note that sequence alignments indicated that the size of the flagellin monomers from the Gram-positive bacterium *Bacillus halodurans*

Alk36 (272 amino acids) is much smaller than that of *S. typhimurium* (494 amino acids) (Crampton *et al.*, 2007). This difference in size suggests that much larger heterologous peptides may potentially be cloned into the *B. halodurans* flagellin protein compared to that of *Salmonella* spp. Consequently, the aims of this part of the study were (i) to construct recombinant expression vectors capable of expressing the *C. perfringens* Type D epsilon toxin and as a protein fusion with either the full-length or a truncated version of *B. halodurans* Alk36 flagellin, (ii) to express the recombinant proteins in *E. coli*, and (iii) to optimize expression of the respective recombinant proteins.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this part of the study are listed in Table 2.1. The *B. halodurans* Alk36 and *E. coli* strains were routinely cultivated in Luria-Bertani (LB) broth (0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7) at 37°C with shaking at 200 rpm, and maintained at 4°C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70°C as glycerol cultures. For recombinant protein expression, *E. coli* strains were also cultivated in EnPresso™ medium (BioSilta), which was prepared according to the manufacturer's specifications. For plasmid DNA selection and maintenance in *E. coli*, the concentrations of antibiotics used were 100 µg/ml for ampicillin and 50 µg/ml for kanamycin. All antibiotics were purchased from Sigma-Aldrich.

The pBluescript II SK cloning vector was obtained from Stratagene, and the pET-28a(+) expression vector was obtained from Novagen. Recombinant plasmid pUC-Etx, harbouring a synthetic DNA copy of the *C. perfringens* Type D *etx* gene, was obtained from GenScript Corp. The *etx* gene was codon-optimized for expression in *E. coli* and is flanked by *Bam*HI and *Hind*III restriction endonuclease recognition sequences at the 5' and 3' ends, respectively. Recombinant plasmid pNWNCFFuz harboured a truncated version of the *hag* gene that encodes for *B. halodurans* Alk36 flagellin. This construct, in which 157 amino acids of the flagellin central variable region was deleted, was obtained from Dr E. Berger, BioSciences, CSIR, Pretoria.

Table 2.1 Bacterial strains and plasmids used in this study

Strains or Plasmids	Characteristics	Source or Reference
Strains:		
E. coli DH10B	Cloning host; F⁻ *mrc*A Δ(*mrr-hsdRMS-mcrBC)* ΔlacX74 *end*A1 *rec*A1 *deo*R Δ(*ara-leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ^-	Invitrogen
E. coli BL21-Gold(DE3)	Expression host; F⁻ *ampThsdS* (r_B- m_B-) *dcm*⁺ Tet^r *gal*λ(DE3) *endA* Hte	Novagen
B. halodurans Alk36	Source of *hag* gene	Louw *et al.*, 1993
Plasmids:		
pBluescript II SK	*E. coli* cloning vector; ColE1 *ori*, Amp^r, LacZα-peptide	Stratagene
pET-28a(+)	*E. coli* expression vector; f1 *ori*, pBR322 *ori*, *lacI*, Kan^r, His-tag coding sequence, T7 RNA polymerase promoter	Novagen
pUC-Etx	pUC19 harbouring a synthetic *E. coli* codon-optimized full-length *etx* gene of *C. perfringen*s Type D	GenScript
pNWNCFFuz	pNW33N, σ^D promoter, truncated *hag* gene (Δ*hag*)	Berger *et al.*, 2011
pSK-Etx1	pBluescript II SK harbouring a truncated *etx* gene, lacking the N-terminal nine amino acids (*etx*1)	This study
pSK-Letx1	pBluescript II SK harbouring the truncated *etx*1 gene with linker sequence at the 5'-end	This study
pSK-NC	pBluescript II SK harbouring the truncated *B. halodurans* Alk36 *hag* gene (Δ*hag*)	This study
pSK-Hag	pBluescript II SK harbouring the full-length *B. halodurans* Alk36 *hag* gene	This study
pEtx0	pET-28a(+) harbouring the synthetic codon-optimized *etx* gene from pUC-Etx	This study
pEtx1	pET-28a(+) harbouring the truncated *etx*1 gene from pSK-Etx1	This study
pNCetx1	pET-28a(+) harbouring a fusion gene between the truncated Δ*hag* gene from pSK-NC and the truncated *etx*1 gene from pSK-Letx1	This study
pHagetx1	pET-28a(+) harbouring a fusion gene between the full-length *hag* gene from pSK-Hag and the truncated *etx*1 gene from pSK-Letx1	This study

2.2.2 Genomic DNA extraction

Genomic DNA was isolated from *B. halodurans* strain Alk36, as described by Lovett and Keggins (1979) with the following modifications. The bacterial cells from 200 ml of an overnight culture were harvested by centrifugation at 6000 × *g* for 15 min, rinsed once with TES buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl; pH 7.5) and suspended in 50 ml of the same buffer. Lysozyme was added to the cell suspension to a final concentration of 10 mg/ml, followed by incubation at 37°C for 25 min. The suspension was subsequently diluted with an equal volume of TES buffer, and Proteinase K was added to a final concentration of 100 µg/ml and Sarkosyl to a final concentration of 0.8% (w/v). Following incubation at 37°C for 30 min, the DNA sample was deproteinized by repeated phenol/chloroform (1:1) extractions. The genomic DNA was precipitated from the final aqueous phase by addition of two volumes of ice-cold 96% ethanol and incubation at -20°C overnight. The precipitated genomic DNA was spooled out on a hooked Pasteur pipette, air-dried and suspended in 5 ml of TE buffer (10 mM Tris, 1 mM EDTA; pH 8).

2.2.3 DNA amplification

2.2.3.1 Primers

The primers used in PCR to amplify different DNA fragments for cloning purposes are indicated in Table 2.2. The primers were designed based on the nucleotide sequences of a synthetic codon-optimized *etx* gene from *C. perfringens* Type D (as supplied by GenScript Corp.) and the *hag* gene from *B. halodurans* Alk36 (Crampton *et al.*, 2007). To facilitate cloning of the amplicons, unique restriction endonuclease recognition sites were incorporated at the 5' terminus of the respective primers. The primers were obtained from Inqaba Biotechnical Industries.

2.2.3.2 Polymerase chain reaction (PCR)

The PCR reaction mixture (50 µl) contained 10 ng of the recombinant plasmid DNA or 50 ng of *B. halodurans* genomic DNA as template, 1 × KAPA HiFi Fidelity buffer, 2 mM $MgCl_2$, 300 µM of each deoxynucleotide triphosphate (dNTP), 0.3 µM of each the appropriate forward and reverse primers and 0.5 U of KAPA HiFi DNA polymerase (Kappa Biosystems). The PCR tubes were placed in a MultiGene™ OptiMax Thermal Cycler (Labnet International, Inc.). Following incubation at 95°C for 2 min, the samples were subjected to 35 cycles of

Table 2.2 Primers used in this study

Primer	Nucleotide sequence	Properties
PCR amplification[*]:		
Optetx1-F	5' - GCG**ggatcc**GCGATCGCGTCTGCGGTTATCTCTATCTAC - 3'	*Bam*HI site
Loptetx2-F[#]	5' - CAG**ctcgag**TCTGGTTCTGGTTCTGGTTCTGCGATCGCGTCTGCGGTTATC - 3'	*Xho*I site
R-epsilon	5' - GCG**aagctt**TTATTTGATACCCGGCGCTTTGAT - 3'	*Hind*IIIsite
HagstartF	5' - CAG**ggatcc**ATTATCAATCACAATTTA - 3'	*Bam*HIsite
HagendR	5' - GCA**ctcgag**ACGAAGTAATTGTAATACAGC - 3'	*Xho*I site
Nucleotide sequencing[§]:		
pUC/M13 Forward	5' - GTAAAACGACGGCCAGT - 3'	
pUC/M13 Reverse	5' - AACAGCTATGACCATG - 3'	
T7 promoter	5' - TAATACGACTCACTATAGGG - 3'	
T7 terminator	5' - GCTAGTTATTGCTCAGCGG - 3'	

* The restriction endonuclease sites are indicated in bold lower case letters
[#] A flexible linker sequence incorporated at the 5'-end of the *etx*1 gene is underlined
[§] The pUC/M13 primers were used for sequencing of the recombinant pBluescript II SK constructs, whereas the T7 promoter and terminator primers were used for sequencing of the recombinant pET-28a(+) constructs

denaturation at 98°C for 20 s, primer annealing at 54°C for 15 s and elongation at 68°C for 30 s. After the last cycle, a final extension step was performed at 68°C for 3 min to complete synthesis of all DNA strands. For control purposes, reaction mixtures containing all reagents except template DNA was included. The PCR reaction mixtures were analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

2.2.4 Agarose gel electrophoresis

DNA was analyzed by agarose gel electrophoresis (Sambrook and Russel, 2001). A horizontal 0.8% (w/v) agarose gel was cast and electrophoresed at 90 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA; pH 8.5). To allow visualization of the DNA when viewed on a UV transilluminator, the gel was supplemented with 0.5 µg/ml ethidium bromide. The size of DNA fragments was determined according to their migration pattern in an agarose gel compared to that of a DNA molecular weight marker (O'GeneRuler™ 1 kb DNA Ladder; Fermentas).

2.2.5 Purification of DNA from agarose gels

The Zymoclean™ Gel DNA Recovery Kit (Zymo Research) was used to extract and purify DNA fragments from agarose gel slices, according to the manufacturer's instructions. Briefly, 3 volumes of ADB buffer was added to each gel slice and incubated at 50°C for 10 min. The melted agarose solutions were then centrifuged through Zymo-Spin™ DNA-binding columns at 10 000 × *g* for 1 min. Each column was subsequently washed twice with 200 µl of Wash buffer and the DNA was eluted in 10 µl of the supplied DNA Elution buffer. An aliquot of each sample was analyzed by agarose gel electrophoresis to assess the purity of the DNA. The concentration of the plasmid DNA was determined with a Beckman Coulter DU® 800 spectrophotometer.

2.2.6 Cloning of DNA fragments into plasmid vectors

For cloning of amplicons, which contained blunt ends due to the use of a proofreading DNA polymerase in the PCR, plasmid pBluescript II SK (Stratagene) was prepared by digestion with *Eco*RV and ligation reactions were performed with the FAST-Link™ DNA ligation kit (Epicentre Technologies). The gel-purified amplicon (40 ng) and blunt-ended vector DNA

(20 ng) were ligated at room temperature for 1 h in a reaction mixture that contained 1 × FAST-Link™ ligation buffer (33 mM Tris-OAc [pH 7.8], 66 mM KOAc, 10 mM $MgCl_2$, 0.5 mM DTT, 10 mM ATP) and 1.5 U of DNA ligase in a final reaction volume of 7.5 µl. For all other ligation reactions, the DNA fragments of interest and restricted vector DNA were ligated by making use of the same protocol.

2.2.7 Transformation of *E. coli*

2.2.7.1 Preparation of electro-competent *E. coli* cells

Electro-competent *E. coli* cells were prepared according to the method of Armitage *et al.* (1988) with the following modifications. An overnight *E. coli* culture was inoculated into 200 ml of pre-warmed (37°C) sterile LB broth to an OD_{600} of 0.05 and incubated at 37°C until an OD_{600} of 0.6 was reached. The culture was subsequently incubated on ice for 30 min and the cells were then harvested by centrifugation at 6000 × *g* for 10 min in a Sorvall RC-5B centrifuge. The cells were rinsed twice with 50 ml of ice-cold 10% (v/v) glycerol and suspended in 0.6 ml of ice-cold GYT medium (10% [v/v] glycerol, 0.125% [w/v] yeast extract, 0.25% [w/v] tryptone; pH 7.3). Aliquots (70 µl) of the cells were pipetted into 1.5-ml Eppendorf tubes and either used immediately or stored at -70°C until further use.

2.2.7.2 Electroporation

DNA was introduced into the electro-competent *E. coli* cells by electroporation according to the method described by Dower *et al.* (1988). For this purpose, an aliquot of the ligation reaction mixture (1 µl) was added to 70 µl of the competent cells, and then transferred to an ice-cold electroporation cuvette with a 0.1-cm electrode gap (BioRad). The cells were exposed to a single electrical pulse using a BioRad Gene-Pulser™ set at 1.8 kV, 25 µF and 200 Ω. Immediately following the electrical discharge, 1 ml of SOC recovery medium (0.5% [w/v] yeast extract, 2% [w/v] tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose; pH 7) was added to the cells. The transformation mixtures were incubated at 37°C for 1 h with shaking and the transformed cells were selected by plating 200-µl aliquots of the cells onto LB agar containing the appropriate antibiotic. The agar plates were incubated at 37°C overnight. When appropriate, the cells were plated together with 10 µl of IPTG (100 mM stock solution) and 50 µl of X-gal (2% [w/v] stock solution) to

allow for blue/white colour selection, based on insertional inactivation of the *lacZ'* marker gene in the pBluescript II SK vector.

2.2.8 Screening and characterization of transformants

2.2.8.1 Plasmid DNA extraction

Plasmid DNA was extracted with the alkaline lysis method, as described by Sambrook and Russel (2001). Single colonies were each inoculated into 5 ml of LB broth supplemented with the appropriate antibiotic and incubated at 37°C overnight. The cells from 2 ml of the overnight cultures were harvested by centrifugation at 10 000 × *g* for 10 min, and the cell pellets were suspended in 200 µl of ice-cold Solution 1 (50 mM glucose, 25 mM Tris-HCl [pH 7.5], 10 mM EDTA, 100 µg/ml RNase A). The cells were lysed by addition of 400 µl of Solution 2 (0.2 N NaOH, 1% [w/v] SDS) and after incubation on ice for 5 min, 300 µl of ice-cold Solution 3 (2.55 M KOAc; pH 4.8) was added. After incubation on ice for 10 min, the insoluble aggregate that formed was removed by centrifugation at 10 000 × *g* for 10 min. The plasmid DNA was precipitated from the recovered supernatant by addition of 450 µl of isopropanol and collected by centrifugation at 15 000 × *g* for 10 min. The plasmid DNA was rinsed once with 70% ethanol, air-dried and suspended in 20 µl of TE buffer.

In addition to the above protocol, plasmid DNA was also extracted and purified using a Zyppy™ Plasmid Miniprep kit (Zymo Research) according to the manufacturer's instructions. Briefly, 600 µl of an overnight culture was mixed with 100 µl of 7× Lysis buffer and then 450 µl of Neutralization buffer was added. Following centrifugation at 15 000 × *g* for 4 min, the supernatant was transferred into a Zymo-Spin™ IIN DNA-binding column and centrifuged for 15 s. The column was subsequently washed twice with Wash buffer and the plasmid DNA was then eluted with 30 µl of the supplied Zippy™ Elution buffer.

2.2.8.2 Colony-PCR

Since the pET-28a(+) expression vector does not allow for blue/white selection, recombinant transformants derived from these cloning experiments were identified by performing a colony-PCR on randomly selected colonies. The colonies from replica agar plates were each suspended in 10 µl of sterile distilled water, vortexed and boiled at 95°C for 5 min. The cellular debris was pelleted by centrifugation at 13 000 × *g* for 2 min and 4 µl of the

supernatant, containing the DNA, was used as template in PCR reactions. In addition to the cleared cell lysate, the PCR reaction mixtures (25 µl) contained 1 × KAPA2G buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each the appropriate forward and reverse primers (Table 2.1), and 1 U of KAPA2G Fast DNA polymerase (Kappa Biosystems). The PCR tubes were placed in a MultiGene™ OptiMax Thermal Cycler (Labnet International, Inc.) and the thermocycling parameters were as follows: initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 10 s, primer annealing at 54°C for 10 s, elongation at 72°C for 15 s, and a final extension at 72°C for 3 min. Aliquots of the PCR reaction mixtures were analyzed by electrophoresis on a 0.8% (w/v) agarose gel in the presence of a DNA molecular weight marker.

2.2.8.3 Restriction endonuclease digestions

Approximately 1 to 2 µg of plasmid DNA was digested with 2 U of enzyme in the appropriate concentration of salt (using the 10× buffer supplied by the manufacturer). The reaction volumes were typically 50 µl and incubation was at 37°C for 1 h. For digestions involving two different restriction enzymes, the buffer that results in the highest activity for both enzymes was selected for use in the digestion reactions. All the restriction endonucleases were supplied by Fermentas. Following digestion, the restriction products were analyzed by agarose gel electrophoresis in the presence of a DNA molecular weight marker.

2.2.8.4 Nucleotide sequencing and analysis

Recombinant plasmid DNA was submitted to Inqaba Biotechnical Industries for nucleotide sequencing on an Applied Biosystems® Model 3500XL automated DNA sequencer. The nucleotide sequences obtained were analyzed with FinchTV v.1.4 (Geospiza, Inc.) and DNAMAN v.4.11 (Lynnon Biosoft) software. The nucleotide and deduced amino acid sequences were compared against entries in the GenBank database with the BLAST-N and BLAST-P programs (Altschul *et al*., 1997), respectively, available on the National Centre for Biotechnology Information web page (http://www.ncbi.nlm.nih.gov/). Pairwise alignments of nucleotide and deduced amino acid sequences were performed with the PRALINE multiple sequence alignment programme (http://www.ibi.vu.nl/programs/pralinewww/).

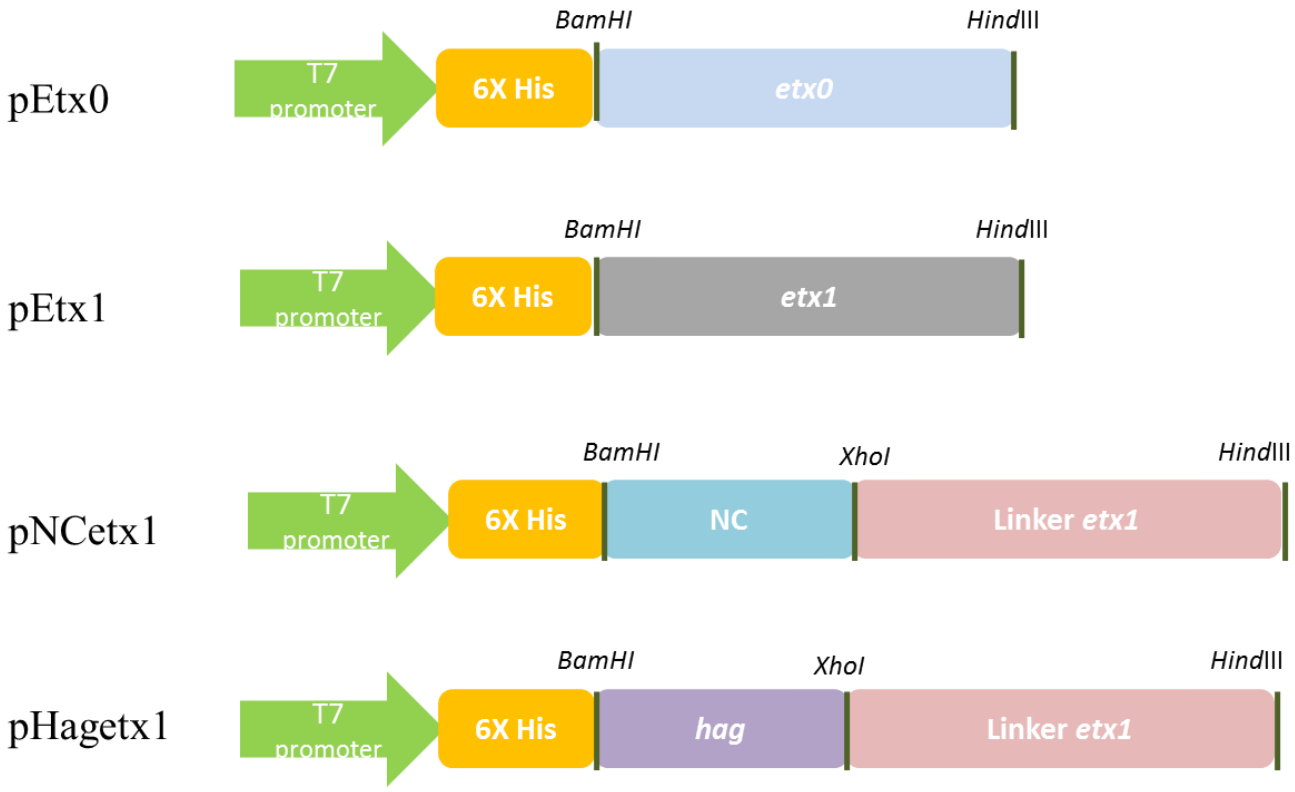


Fig. 2.1 Schematic representation of expression constructs generated during the course of this study. The constructs were cloned directionally under transcriptional control of the T7 RNA polymerase promoter in the pET-28a(+) bacterial expression vector, and was cloned in-frame to allow for expression of the recombinant proteins as N-terminal histidine-tagged proteins. In the figure, *etx0* represents the full-length epsilon gene, inclusive of the N-terminal signal peptide sequence; *etx1* represents a truncated *etx* gene lacking the first nine amino acids of the signal peptide sequence; *hag* represents the *B. halodurans* Alk36 flagellin-encoding gene; and NC represents the truncated version of the *hag* gene that lacks sequences encoding 157 amino acids of the flagellin variable region. In the pNCetx1 and pHagetx1 plasmids, the full-length and truncated *hag* genes were each fused to the *etx*1 gene that was modified through the incorporation of a flexible linker sequence at its 5'-end.

2.2.9 Plasmid constructs

All molecular cloning techniques used in the construction of the different recombinant plasmids were performed in accordance to the procedures described in the preceding sections. All plasmid constructs were confirmed by agarose gel electrophoresis following restriction endonuclease digestions and by nucleotide sequencing. A schematic representation of the constructed expression cassettes is presented in Fig. 2.1.

- **pEtx0**

The full-length chemically synthesized *etx* gene was excised from plasmid pUC-Etx by digestion with both *Bam*HI and *Hind*III and the insert DNA was then cloned into the *Bam*HI and *Hind*III sites of the pET-28a(+) expression vector to generate pEtx0.

- **pEtx1**

A truncated *etx* gene, lacking the N-terminal nine amino acids (MKKNLVKSL), was generated by PCR using plasmid pUC-Etx as template DNA together with primers Optetx1-F and R-epsilon. The amplicon was blunt-end cloned into the *Eco*RV site of plasmid pBluescript II SK to generate pSK-Etx1. The insert DNA was subsequently recovered by digestion with both *Bam*HI and *Hind*III and then cloned into identically digested pET-28a(+) to generate pEtx1.

- **pNCetx1**

In constructing the recombinant expression vector pNCetx1, the truncated *etx*1 gene was modified through the incorporation of a seven-amino-acid linker sequence (SGSGSGS) at its N terminus. For this purpose, plasmid pUC-Etx was used as template DNA in the PCR together with primers Loptetx2-F and R-epsilon, which contain a *Xho*I and *Hind*III site, respectively. The amplicon was blunt-end cloned into the *Eco*RV site of plasmid pBluescript II SK to generate pSK-Letx1. Primers HagstartF and HagendR, which contain a *Bam*HI and *Xho*I site, respectively, were subsequently used with plasmid pNWNCFFuz as template DNA to PCR-amplify a truncated *hag* gene that lacks the variable region (Δ*hag*). The amplicon was cloned into the *Eco*RV site of plasmid pBluescript II SK to generate pSK-NC. The cloned DNA fragments were subsequently recovered by restriction enzyme digestion of plasmid pSK-Letx1 with both *Xho*I and *Hind*III, and plasmid pSK-NC with both *Xho*I and *Bam*HI. These two DNA fragments were then used in a three-way ligation, together with

pET-28a(+) that had been digested with both *Bam*HI and *Hind*III, to complete construction of pNCetx1.

- **pHagetx1**

Primers HagstartF and HagendR were used together with genomic DNA of *B. halodurans* Alk36 as template DNA to PCR amplify the full-length *hag* gene. The amplicon was blunt-end cloned into the *Eco*RV site of plasmid pBluescript II SK to generate pSK-Hag. The *hag* gene was subsequently excised from the recombinant plasmid DNA by digestion with both *Bam*HI and *Xho*I, and the modified truncated *etx*1 gene was excised from pSK-Letx1 with both *Xho*I and *Hind*III. Using a cloning strategy similar to that described above, the respective DNA fragments were used in a three-way ligation with *Bam*HI and *Hind*III-digested pET-28a(+) to generate pHagetx1.

2.2.10 Expression of recombinant proteins in *E. coli*

Single colonies of *E. coli* BL21-Gold(DE3) transformed with either recombinant or parental pET-28a(+) plasmid DNA were inoculated into 5 ml of LB medium containing kanamycin and incubated at 37°C overnight with shaking. Expression of the recombinant proteins were induced with IPTG. For this purpose, the overnight cultures were inoculated into 25 ml of pre-warmed (37°C) sterile LB medium to an OD_{600} of 0.1 and incubated at 37°C until an OD_{600} of 0.6 was reached. To each culture, IPTG was added to a final concentration of 1 mM and the cultures were incubated at 30°C with shaking at 200 rpm for an additional 6 h. The bacterial cells were subsequently harvested by centrifugation at 7000 × *g* for 10 min at 4°C. The cell-free culture supernatant (extracellular protein fraction) was retained. The cell pellet was suspended in 25 ml of 1 × Phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 1.4 mM KH_2PO_4; pH 7.5) and 0.5 ml of the suspension was transferred into a 2-ml Eppendorf tube. The cells were lysed by sonication on ice for 3 min using a Bendelin Sonopuls at a power output of 67%. The cell lysate was clarified by centrifugation (16 000 × *g*, 15 min) and the supernatant, considered the soluble cytoplasmic protein fraction, was recovered. The pellet (insoluble cytoplasmic protein fraction) was suspended in 0.5 ml of 1 × PBS buffer (pH 7.5).

2.2.11 Periplasmic protein extraction

Periplasmic protein fractions were prepared by making use of an osmotic shock protocol (Ausubel *et al.*, 1989). *E. coli* BL21-Gold(DE3) cells transformed with recombinant plasmid pEtx0 was cultivated in EnPresso™ medium and induced with 1 mM IPTG for 2 h to allow expression of the recombinant protein. The cells from 50 ml of the culture were harvested by centrifugation at 6000 × *g* for 10 min at 4ºC. The cell pellet was suspended in 25 ml of 30 mM Tris-HCl (pH 8) containing 20% (w/v) sucrose, and then 0.5 M EDTA (pH 8) was added to a final concentration of 0.1 mM. The suspension was incubated at room temperature for 10 min with slow stirring (50 rpm), after which the cells were collected by centrifugation at 10 000 × *g* for 10 min at 4ºC. The supernatant was discarded and the cell pellet was suspended gently in 25 ml of ice-cold 5 mM $MgSO_4$, followed by incubation on ice for 10 min with slow stirring to allow release of the periplasmic proteins into the buffer. Following centrifugation (10 000 × *g*, 10 min, 4ºC), the supernatant, termed the periplasmic protein fraction, was recovered and stored at -20ºC until use. The cell pellet was suspended in 25 ml of 1 × PBS (pH 7.5) and lysed by sonication on ice for 5 min. The lysate was centrifuged and the supernatant containing soluble cytoplasmic proteins was retained. Proper cell fractionation was assessed by Western blot analysis with an antibody to the cytoplasmic GroEL protein, as described below (Section 2.2.12.2).

2.2.12 Analysis of recombinant proteins

2.2.12.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to analysis, protein samples were mixed with an equal volume of 2 × Protein Solvent Buffer (PSB: 125 mM Tris-HCl [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue) and then denatured by heating to 95°C for 5 min in a boiling water bath. The proteins were resolved by electrophoresis in a discontinuous gel system, as described by Laemmli (1970). A 5% (w/v) acrylamide stacking gel (5% [w/v] acrylamide, 0.17% [w/v] bis-acrylamide, 125 mM Tris-HCl [pH 6.8], 0.1% [w/v] SDS) and a 12% (w/v) acrylamide separating gel (12% [w/v] acrylamide, 0.34% [w/v] bis-acrylamide, 0.375 mM Tris-HCl [pH 8.8], 0.1% [w/v] SDS) were polymerized by addition of 0.08% (w/v) ammonium persulphate and 10 µl of TEMED. Electrophoresis was performed in a Mini-PROTEAN® TetraCell system (BioRad) at 100 V for 1 h in 1 × TGS electrophoresis buffer (0.025 M Tris-HCl [pH 8.3], 0.192 M glycine, 0.1% [w/v] SDS). Following

electrophoresis, the gels were stained for 30 min with 0.125% (w/v) Coomassie brilliant blue G-250 (prepared in 50% [v/v] methanol and 10% [w/v] acetic acid), and then destained in a solution containing 25% (v/v) methanol and 10% (v/v) glacial acetic acid until the proteins were visible. The sizes of the resolved proteins were estimated by comparison to reference molecular mass proteins (PageRuler™ Plus Pre-stained Protein Ladder; Fermentas).

2.2.12.2 Western blot analysis

For Western blot analysis (Gallagher *et al.*, 2004), proteins from an unstained SDS-polyacrylamide gel were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Two sheets of filter paper and the PVDF membrane, cut to the same size as the gel, were equilibrated for 10 min in transfer buffer (0.025 M Tris-HCl [pH 8.3], 0.192 M glycine, 0.1% [w/v] SDS, 10% methanol). The proteins were electroblotted onto the membrane at 5 V for 16 h with a Trans-Blot® SD blotting apparatus (BioRad). Following transfer, the membrane was rinsed in 1 × PBS for 5 min and non-specific binding sites were blocked by immersing the membrane for 1 h in blocking solution (3% [w/v] fat-free milk powder, 10 mM Tris [pH 7.5], 150 mM NaCl, 0.1%[v/v] Tween-20). The membrane was washed for 5 min and then transferred to blocking solution containing the primary antibody. These comprised either of a polyclonal anti-epsilon antibody (Onderstepoort Biological Products, Pretoria) or monoclonal anti-GroEL antibody (Sigma-Aldrich), which were diluted 1:1000 in blocking solution. Following incubation at room temperature for 2 h with gentle agitation, the unbound primary antibodies were removed by washing the membrane three times for 5 min each with wash buffer. An appropriate secondary antibody was then added to the membrane. These comprised of anti-guinea pig IgG conjugated to horseradish peroxidase (Abcam) for detection of the epsilon toxin or anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich) for detection of GroEL. The respective secondary antibodies were diluted 1:2000 and 1:4000 in blocking solution. Following incubation at room temperature for 1 h with gentle agitation, the membrane was washed three times for 5 min each with wash buffer and once with 1 × PBS for 5 min. To detect immune-reactive proteins, a 5,5'-tetramethylbenzidine (TMB) solution (Sigma-Aldrich) was added to the membrane, followed by incubation until the protein bands became visible. The colour detection reaction was then stopped by addition of 0.5 M EDTA.

2.2.13 Optimization of recombinant protein expression in *E. coli*

To optimize the expression of recombinant proteins in *E. coli* BL21-Gold(DE3) cultures, different cultivation media (LB and EnPresso™) were evaluated and different concentrations of the inducer were assessed. For media evaluation, recombinant cultures were grown in 500-ml baffled flasks containing LB or EnPresso™ medium and protein expression was induced with 1 mM IPTG. For expression in LB medium, the methodology, as described in Section 2.2.10, was followed. For expression in the EnPresso™ cultivation medium, a pre-culture was prepared by inoculating 5 ml of LB broth with 100 µl of the glycerol bacterial culture. Following incubation at 37°C for 7 h with shaking at 200 rpm, 500 µl of the bacterial culture was transferred to 50 ml of EnPresso™ broth in 500-ml baffled flasks and 0.3 U of EnZ I'm (BioSilta) was added to the flask. The flask was sealed with Steri-Wrap® and incubated at 30°C for 16 h with shaking. After incubation, the culture was supplemented with a booster tablet, supplied with the medium, and 0.6 U of EnZ I'm was added. The culture was simultaneously induced for recombinant protein expression by addition of IPTG to a final concentration of 1 mM. The cultures grown in EnPresso™ medium were incubated at 30°C for 24 h as recommended by the manufacturer, whereas those grown in LB medium were incubated at 30°C for 20 h based on previous experiments (M. Crampton, personal communication). The cell densities at the end of the induction period were determined by optical density (OD_{600}) measurements using a Beckman Coulter DU® 800 spectrophotometer. To determine the optimal IPTG concentration for induction of recombinant protein synthesis, cultures grown in EnPresso™ medium were induced with IPTG at a final concentration of 0.75, 0.5, 0.25 or 0.1 mM, and the protein concentration of the recombinant proteins was determined at 24 h post-induction.

2.2.14 Protein concentration determination

Densitometric analysis of Coomassie blue-stained SDS-polyacrylamide gels was used to determine the concentrations of the epsilon toxin and flagellin-toxin fusion proteins, as described by Berger *et al.* (2011). The gel pictures were captured using a SynGene G-Box transilluminator and GeneSnap v.7.01 software, after which the protein concentrations were determined with Gene Tools v.3.09 software (SynGene). Bovine serum albumin (BSA; BioRad), at concentrations of 100, 200 and 400 ng/µl, was used to generate a standard curve

and peak areas of the relevant protein bands were then used to calculate the protein concentration derived from the standard curve.

2.3 RESULTS

2.3.1 Construction of recombinant pET-28a(+) bacterial expression vectors

E. coli has been used frequently as a prokaryotic host for expression of the *C. perfringens* Type D epsilon toxin (Hunter *et al.*, 1992; Goswami *et al.*, 1996; Souza *et al.*, 2010; Zhao *et al.*, 2011). Expression of the native gene sequence, however, has been met with technical difficulties, including the accumulation of the recombinant protein in insoluble inclusion bodies and low levels of the expressed protein product. The latter may be a consequence of the sequence containing a number of codons that are rarely employed in *E. coli* genes, which therefore may lead to poor expression (Hunter *et al.*, 1992; Angov *et al.*, 2011). Thus, to circumvent these problems and to improve expression levels, a synthetic codon-optimized gene of the *C. perfringens* Type D epsilon gene (*etx*) was designed. The *etx* gene sequence (GenBank Accession no. AY858558; Chen *et al.*, unpublished) was submitted online to http://genomes.urv.es/OPTIMIZER/optimized.php in order to generate an *E. coli* codon-optimized gene sequence, and this gene sequence was subsequently submitted to GenScript Corp. for chemical synthesis. The synthetic gene was synthesized to also provide terminal *Bam*HI- and *Hind*III-compatible ends and then cloned into pUC19 to generate the recombinant plasmid pUC-Etx. As a final confirmation regarding the integrity of the cloned insert DNA, the nucleotide sequence of the cloned insert DNA was also determined (GenScript Corp.). This construct served as the source of the *etx* gene used in the construction of the various bacterial expression vectors during the course of this study.

2.3.1.1 Construction of expression vectors harbouring different versions of the *etx* gene

Previous studies have shown that a truncated *etx* gene of *C. perfringens* Type D, which lacked the N-terminal nine amino acids, could be expressed successfully in *E. coli* and the purified recombinant protein was shown to elicit high antibody titers in immunized rabbits, cattle, goats and sheep (Lobato *et al.*, 2010; Souza *et al.*, 2010). Notably, the truncated epsilon toxin was also reported to be considerably less toxic than the native toxin. This is advantageous since the truncated epsilon protein could be used without the need for additional pre-treatments in order to reduce its toxicity (Souza *et al.*, 2010). For comparative analyses, both

the full-length and a similarly truncated *etx* gene were therefore cloned and expressed during the course of this study.

- **Construction of pEtx0 harbouring the full-length *etx* gene**

To construct the recombinant expression plasmid pEtx0, the insert DNA was recovered from pUC-Etx by digestion with both *Bam*HI and *Hind*III and ligated with similarly prepared pET-28a(+) vector DNA, thereby allowing for the directional cloning of the insert DNA. Following electroporation of *E. coli* DH10B cells with the ligation reaction mixture, a number of kanamycin-resistant transformants were selected randomly and replica-plated. The transformants were initially screened for the presence of insert DNA by colony-PCR with the T7 promoter and T7 terminator primers, which flank the multiple cloning site of the pET-28a(+) vector. Transformants that yielded an expected product of 996 bp were selected (results not shown), and the plasmid DNA was extracted. To confirm successful construction of pEtx0, the plasmid DNA was digested with both *Bam*HI and *Hind*III. Agarose gel electrophoresis of the digestion products yielded DNA fragments corresponding with the size of the vector DNA (ca. 5.37 kb) and the full-length *etx* gene (996 bp) (Fig. 2.2, lane 7). The integrity of the *etx* gene and frame position was verified by nucleotide sequencing of the cloned insert DNA, and the sequence is provided in the Appendix to this dissertation.

- **Construction of pEtx1 harbouring a truncated *etx* gene**

Using plasmid pUC-Etx as template DNA, PCR amplification was performed with primers Optetx1-F (containing a *Bam*HI site) and R-epsilon (containing a *Hind*III site), as described under Materials and Methods (Section 2.2.9). An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single amplicon of the expected size (963 bp) was observed (Fig. 2.3, lane 3). In contrast, no amplification products were observed in the negative control reaction mixture in which template DNA was omitted (Fig. 2.3, lane 2). The amplicon was subsequently purified from the agarose gel and blunt-end cloned into pBluescript II SK vector DNA. Following electroporation of *E. coli* DH10B cells, recombinant transformants with a Gal^- phenotype were selected from X-gal containing indicator plates and cultured in LB broth supplemented with ampicillin. The extracted plasmid DNA was analyzed by agarose gel electrophoresis and by restriction enzyme digestion. Plasmid DNA migrating slower than the parental pBluescript II SK vector DNA on agarose gels were selected and analyzed for the presence of a cloned insert DNA by digestion

with both *Bam*HI and *Hind*III. Following agarose gel electrophoresis, restriction fragments of ca. 3.0 kb and 963 bp, respectively, were observed, which is in agreement with the expected size of the pBluescript II SK vector DNA and insert DNA (Fig 2.3 lane 7). A recombinant clone, designated pSK-Etx1, was selected and used in further DNA manipulations.

To complete construction of the recombinant bacterial expression plasmid pEtx1, the truncated *etx*1 gene was excised from plasmid pSK-Etx1 and cloned directionally into the *Bam*HI and *Hind*III sites of the pET-28a(+) vector DNA. Kanamycin-resistant transformants resulting from electroporation of *E. coli* DH10B cells were selected randomly and replica-plated. The transformants were initially screened by colony-PCR to confirm the presence of insert DNA by making use of the upstream T7 promoter primer and the downstream gene-specific R-epsilon primer (results not shown). Subsequently, plasmid DNA was extracted from selected transformants and characterized by restriction enzyme digestion. A recombinant plasmid, from which a 963 bp *etx*1-specific (Fig. 2.3, lane 11) insert DNA was

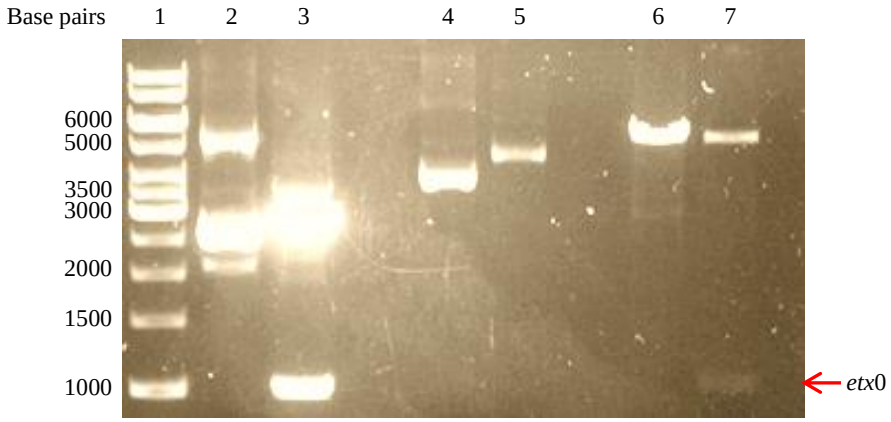


Fig. 2.2 Agarose gel electrophoretic analysis of the recombinant plasmid pEtx0. Lane 1, DNA molecular weight marker; lane 2, uncut pUC-Etx plasmid DNA; lane 3, pUC-Etx plasmid DNA digested with both *Bam*HI and *Hind*III; lane 4, uncut parental pET-28a(+) vector DNA; lane 5, uncut recombinant plasmid pEtx0; lane 6, pET-28a(+) vector DNA digested with both *Bam*HI and *Hind*III; lane 7, pEtx0 plasmid DNA digested with both *Bam*HI and *Hind*III. The sizes of the DNA molecular weight marker, O'GeneRuler™ 1kb DNA Ladder (Fermentas), are indicated to the left of the figure.

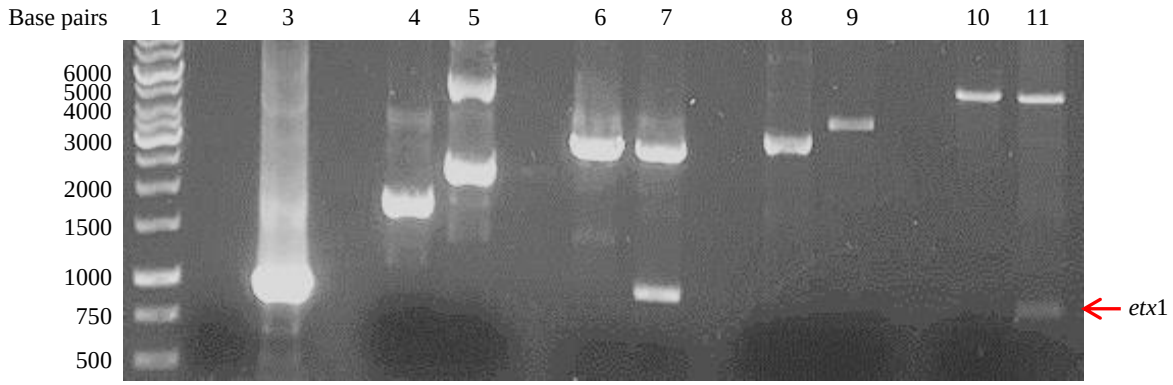


Fig. 2.3 Agarose gel electrophoretic analysis of recombinant plasmid pEtx1, and the intermediate plasmid pSK-Etx1 used in its construction. Lane 1, DNA molecular weight marker; lane 2, negative control PCR reaction mixture lacking template DNA; lane 3, amplicon obtained by PCR amplification using pUC-Etx plasmid DNA as template and primers Optetx1-F and R-epsilon; lane 4, uncut parental pBluescript II SK vector DNA; lane 5, uncut recombinant plasmid pSK-Etx1; lane 6, pBluescript II SK vector DNA linearized with *Eco*RV; lane 7, pSK-Etx1 plasmid DNA digested with both *Bam*HI and *Hind*III; lane 8, uncut parental pET-28a(+) vector DNA; lane 9, uncut recombinant plasmid pEtx1; lane 10, pET-28a(+) vector DNA digested with both *Bam*HI and *Hind*III; lane 11, pEtx1 plasmid DNA digested with both *Bam*HI and *Hind*III. The sizes of the DNA molecular weight marker, O'GeneRuler™ 1kb DNA Ladder (Fermentas), are indicated to the left of the figure.

excised, was selected for further use and designated pEtx1. The integrity of the cloned insert DNA was verified by nucleotide sequencing, the result of which confirmed that nucleotides specifying the N-terminal nine amino acids were successfully deleted. In addition, analysis of the deduced amino acid sequence confirmed that the open reading frame of the protein was maintained. The complete nucleotide sequence of the cloned *etx*1 gene is provided in the Appendix to this dissertation.

2.3.1.2 Construction of expression vectors harbouring fusion genes

Previous reports have demonstrated that fusing a protein antigen to the TLR5 ligand, flagellin, markedly enhanced the immunogenicity of the antigen and induced antigen-specific immune responses *in vivo* (Huleatt *et al.*, 2007; McDonald *et al.*, 2007). Based on the lack of knowledge regarding the adjuvant activity of flagellin from Gram-positive bacteria, this study aimed at providing fusion proteins whereby the adjuvant activity of Gram-positive bacterial flagellin could be evaluated in the context of a vaccine against *C. perfringens* Type D enterotoxemia. For this purpose, constructs were prepared that contained the full-length or a truncated *B. halodurans* Alk36 *hag* gene fused to the *etx*1 gene. Flagellin proteins with truncated hypervariable regions have been used with success as fusion partners in previous studies and allowed for genetic linkage of larger antigens compared to the use of full-length flagellin proteins (McDonald *et al.*, 2007; Mizel *et al.*, 2009; Ghose *et al.*, 2013). In this study, the *etx*1 gene was also modified through the incorporation of a flexible linker sequence at its 5'-end that encoded for a seven-amino-acid sequence (SGSGSGS). The inclusion of this linker sequence has been proposed to allow for efficient folding of recombinant flagellin-antigen fusion proteins (Song *et al.*, 2008).

- **Construction of pNCetx1 harbouring a Δ*hag*::*etx*1 fusion gene**

To construct the fusion gene, a strategy was followed in which the *etx*1 gene was modified through the inclusion of nucleotides specifying a seven-amino-acid linker sequence (SGSGSGS) at its 5'-end prior to fusing it to the 3'-end of the Δ*hag* gene. To ensure in-frame fusion of the Δ*hag* and *etx*1 genes, the Δ*hag* gene was PCR-amplified using a 3'-specific primer that was designed to anneal upstream of the stop codon. In addition, the 5'-specific primer used to PCR-amplify the *etx*1 gene and the 3'-specific primer used to PCR-amplify the Δ*hag* gene were designed to incorporate a unique *Xho*I restriction endonuclease recognition

site at their ends. Thus, following restriction enzyme digestion of the respective DNA fragments, they could be ligated to yield the desired Δ*hag*::*etx*1 fusion gene.

Towards construction of the Δ*hag*::*etx*1 fusion gene, a truncated *B. halodurans* Alk36 *hag* gene was PCR-amplified using pNWNCFFuz as template DNA and the *etx*1 gene was PCR-amplified using pUC-Etx as template DNA, as described under Materials and Methods (Section 2.2.9). An aliquot of each reaction mixture was analyzed by agarose gel electrophoresis and a single amplicon of the expected size for the Δ*hag* (366 bp) and modified *etx*1 (984 bp) genes were observed. No amplification products were observed in the negative control reactions in which template DNA was omitted (Fig. 2.4, lanes 2 to 5). The agarose gel-purified amplicons were subsequently cloned into the pBluescript II SK vector, as described in the preceding section. To confirm successful cloning of the PCR products, the extracted plasmid DNA was digested with restriction enzymes of which the recognition sequence had been incorporated during the design of the primers. Digestion of the derived recombinant plasmid pSK-NC with both *Xho*I and *Bam*HI yielded DNA fragments corresponding with the size of the vector DNA (ca. 3.0 kb) and the 366-bp Δ*hag* gene (Fig. 2.4, lane 10), whereas digestion of recombinant plasmid pSK-Letx1 with both *Xho*I and *Hind*III resulted in excision of the *etx*1 gene (984 bp) (Fig. 2.4, lane 11). These results therefore confirmed that the Δ*hag* and *etx*1 genes had been cloned successfully into the pBluescript II SK vector DNA. These recombinant plasmids were subsequently used as sources in the construction of a recombinant pET-28a(+) bacterial expression vector containing the Δ*hag*::*etx*1 fusion gene.

To construct the desired Δ*hag*::*etx*1 fusion gene, the Δ*hag* and *etx*1 genes were excised from pSK-NC and pSK-Letx1, respectively, with the appropriate restriction enzymes and the gel-purified DNA fragments were used in a three-way ligation with pET-28a(+) vector DNA that been digested with both *Bam*HI and *Hind*III. Following electroporation of *E. coli* DH10 cells, a number of randomly selected kanamycin-resistant transformants were screened by colony-PCR for the presence of insert DNA with the T7 promoter primer, which anneals upstream of the vector-borne T7 RNA polymerase promoter, and the R-epsilon primer, which anneals at the 3'-end of the fusion gene (results not shown). The plasmid DNA was extracted from selected transformants and characterized by restriction enzyme digestion. Digestion of the recombinant plasmid DNA with both *Bam*HI and *Hind*III yielded two DNA fragments that corresponded in size to the pET-28a(+) vector DNA (5.37 kb) and the cloned Δ*hag*::*etx*1

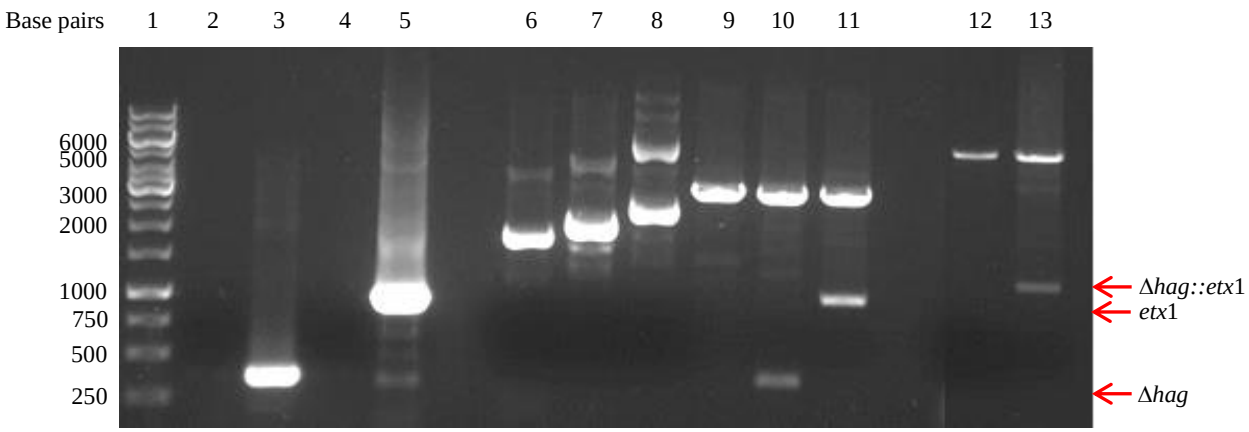


Fig. 2.4 Agarose gel electrophoretic analysis of the recombinant plasmid pNCetx1, and the intermediate plasmids pSK-NC and pSK-Letx1 used in its construction. Lane 1, DNA molecular weight marker; lane 2, negative control PCR reaction mixture lacking template DNA; lane 3, amplicon obtained by PCR amplification using pNWNCFFuz plasmid DNA as template and primers HagstartF and HagendR; lane 4, negative control PCR reaction mixture lacking template DNA; lane 5, amplicon obtained by PCR amplification using pUC-Etx plasmid DNA as template and primers Loptetx2-F and R-epsilon; lane 6, uncut parental pBluscript II SK vector DNA; lane 7, uncut pSK-NC plasmid DNA; lane 8, uncut pSK-Letx1 plasmid DNA; lane 9, pBluescript II SK vector DNA linearized with *Eco*RV; lane 10, pSK-NC plasmid DNA digested with both *Bam*HI and *Xho*I; lane 11, pSK-Letx1 plasmid DNA digested with both *Xho*I and *Hind*III; lane 12, pET-28a(+) vector DNA digested with both *Bam*HI and *Hind*III; lane 13, pNCetx1 plasmid DNA digested with both *Bam*HI and *Hind*III. The sizes of the DNA molecular weight marker, O'GeneRuler™ 1kb DNA Ladder (Fermentas), are indicated to the left of the figure.

fusion gene (1.350 kb) (Fig. 2.4, lane 13), indicating that the fusion gene had been constructed successfully in the bacterial expression vector. The integrity of the cloned insert DNA was verified by nucleotide sequencing, the result of which confirmed that the 5'-end of the *etx*1 gene was genetically linked to the 3'-end of the *hag* gene and these sequences were separated by nucleotides encoding the linker sequence. In addition, analysis of the deduced amino acid sequence confirmed that the open reading frame of the fusion protein was maintained. The complete nucleotide sequence of the cloned Δ*hag*::*etx*1 fusion gene is provided in the Appendix to this dissertation.

- **Construction of pHagetx1 harbouring a *hag*::*etx*1 fusion gene**

To construct a fusion gene composed of the full-length *hag* gene and *etx*1, an identical strategy to that described above was followed, except that genomic DNA of *B. halodurans* Alk36 was used as template in the PCR reaction. The 816-bp amplicon was cloned into pBluescript II SK vector DNA and a recombinant plasmid, from which the *hag*-specific insert DNA was excised, was selected and designated pSK-Hag (Fig. 2.5, lane 10). To construct the *hag*::*etx*1 fusion gene, the *hag* gene was excised from pSK-Hag by digestion with both *Bam*HI and *Xho*I and the *etx*1 gene was recovered from pSK-Letx1 by digestion with both *Xho*I and *Hind*III. The gel-purified DNA fragments were then used in a three-way ligation with pET-28a(+) vector DNA, which had been prepared by digestion with both *Bam*HI and *Hind*III. The derived recombinant plasmid, designated pHagetx1, was characterized by restriction enzyme digestion with *Bam*HI and *Hind*III, and an insert DNA of which the size corresponded with the expected size of the *hag*::*etx*1 fusion gene (1.800 kb) was excised (Fig. 2.5, lane 13).

Although the above results indicated that the fusion gene was successfully constructed in the pET-28a(+) bacterial expression vector, sequence analysis of cloned insert DNA indicated the presence of several nucleotide deletions present at the 5'-end of the *etx*1 gene, as well as in the linker sequence incorporated at the junction of the *hag* and *etx*1 genes. Despite repeating the cloning experiment, similar results were obtained. To overcome this impediment, the cloning strategy was revised and the fusion gene was subsequently constructed without the linker sequence. However, sequence analysis of this fusion gene indicated the presence of a base substitution at nucleotide position 790 that resulted in the introduction of a stop codon within the 3'-region of the *hag* gene (results not shown). Consequently, the pHagetx1 construct was excluded from all further analyses.

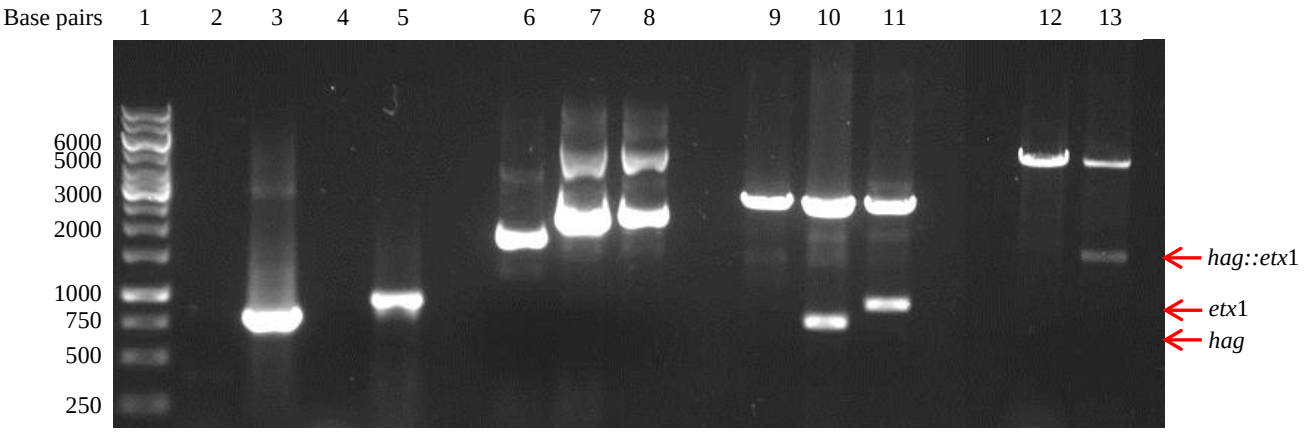


Fig. 2.5 Agarose gel electrophoretic analysis of the recombinant plasmid pHagetx1, and the intermediate plasmids pSK-Hag and pSK-Letx1 used in its construction. Lane 1, DNA molecular weight marker; lane 2, negative control PCR reaction mixture lacking template DNA; lane 3, amplicon obtained by PCR amplification using *B. halodurans* Alk36 genomic as template DNA and primers HagstartF and HagendR; lane 4, negative control PCR reaction mixture lacking template DNA; lane 5, amplicon obtained by PCR amplification using pUC-Etx plasmid DNA as template and primers Loptetx2-F and R-epsilon; lane 6, uncut parental pBluscript II SK vector DNA; lane 7, uncut pSK-Hag plasmid DNA; lane 8, uncut pSK-Letx1 plasmid DNA; lane 9, pBluescript II SK vector DNA linearized with *Eco*RV; lane 10, pSK-Hag plasmid DNA digested with both *Bam*HI and *Xho*I; lane 11, pSK-Letx1 plasmid DNA digested with both *Xho*I and *Hind*III; lane 12, pET-28a(+) vector DNA digested with both *Bam*HI and *Hind*III; lane 13, pHagetx1 plasmid DNA digested with both *Bam*HI and *Hind*III. The sizes of the DNA molecular weight marker, O'GeneRuler™ 1kb DNA Ladder (Fermentas), are indicated to the left of the figure.

2.3.2 Analysis of recombinant protein expression in *E. coli* BL21-Gold(DE3)

2.3.2.1 SDS-PAGE and Western blot analyses

To determine whether the epsilon toxins and flagellin-toxin fusion protein could be expressed in *E. coli*, the parental pET-28a(+) and recombinant pET expression plasmids (pEtx0, pEtx1 and pNCEtx1) were electroporated into *E. coli* BL21-Gold(DE3) cells. For bacterial expression, overnight cultures of the respective bacterial strains were inoculated into LB medium and protein expression was induced with 1 mM IPTG for 6 h. Following incubation, the cells of each culture were harvested, and soluble and insoluble cytoplasmic protein fractions were prepared. These samples were analyzed together with the extracellular protein fractions (cell-free culture supernatants) by SDS-PAGE and Western blot analyses.

Analysis of the Coomassie blue-stained gels indicated the presence of an apparently unique protein in samples prepared from the *E. coli* BL21-Gold(DE3) cells transformed with the pEtx0 (Fig. 2.6a), pEtx1 (Fig. 2.6b) and pNCEtx1 (Fig. 2.6c) recombinant bacterial expression vectors. The molecular mass of the unique proteins was in agreement with the predicted molecular mass of the epsilon toxin lacking a signal peptide sequence (ETX0, 32 kDa), the truncated epsilon toxin (ETX1, 35 kDa), and the ΔHag::Etx1 fusion protein (NC-ETX1, 48 kDa). No similar proteins were observed in the protein samples prepared from *E. coli* BL21-Gold(DE3) cells transformed with the non-recombinant pET-28a(+) vector. To confirm the identity of the proteins, an unstained SDS-polyacrylamide gel was subjected to Western blot analysis. The results indicated that the uniquely expressed proteins were recognized by the polyclonal anti-epsilon antibody, thus confirming successful expression of the respective proteins by the constructed recombinant bacterial expression vectors (Fig. 2.7). In addition, these results also indicate that the respective recombinant proteins retained their antigenicity and, as such, may be useful for evaluation as potential enterotoxemia vaccine candidates.

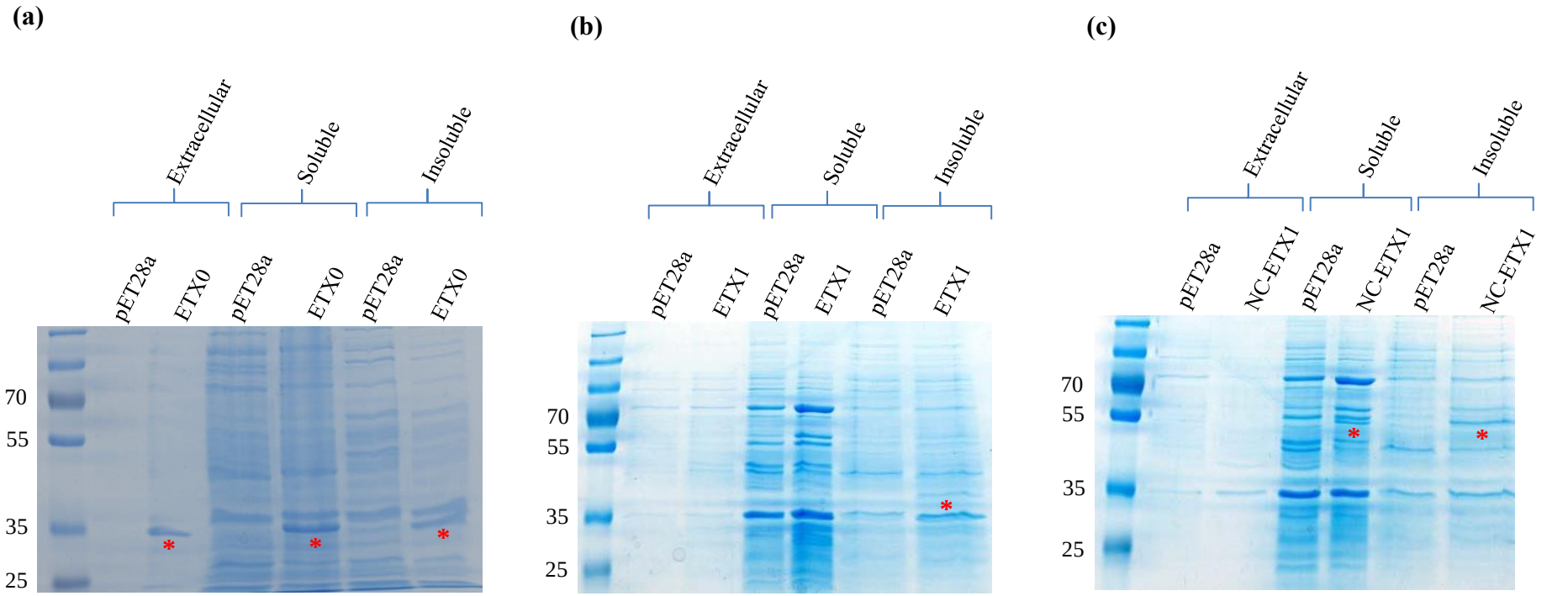
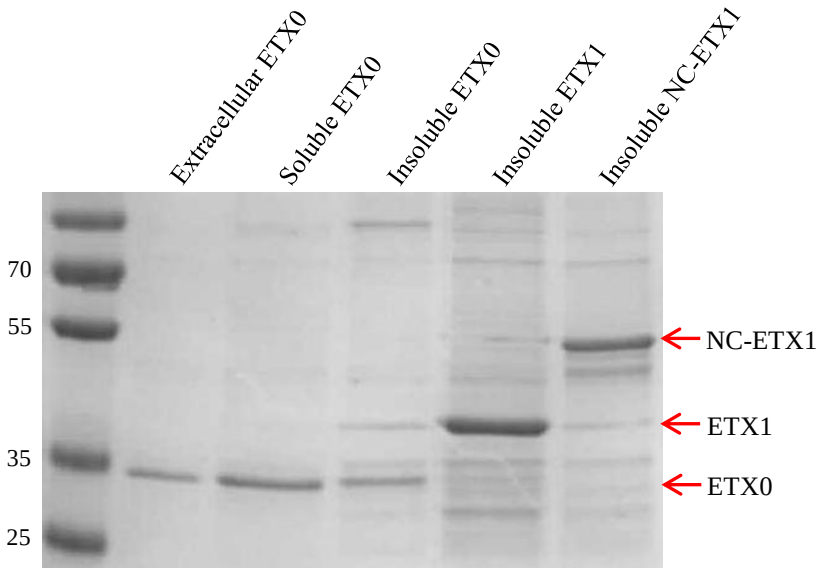


Fig. 2.6 SDS-PAGE analysis of recombinant epsilon toxin and flagellin-toxin proteins expressed in *E. coli* BL21-Gold(DE3). IPTG-induced cultures of *E. coli* BL23-Gold(DE3) cells transformed with recombinant plasmid pEtx0 (a), pEtx1 (b) and pNCEtx1 (c) were harvested 6 h post-induction. IPTG-induced *E. coli* BL23-Gold(DE3) cells transformed with the non-recombinant pET-28a(+) plasmid were included as a control. Following induction, the cells were fractionated and samples of the extracellular protein fraction, as well as soluble and insoluble cytoplasmic protein fractions were resolved by 12% SDS-PAGE. The position of the recombinantETX0, ETX1 and NC-ETX1 proteins are indicated with asterisks. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figures.

(a)



(b)

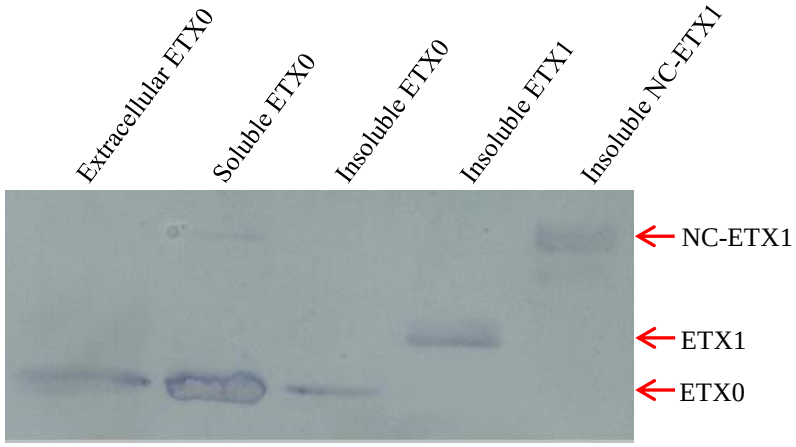


Fig. 2.7 Western blot analysis of recombinant epsilon toxin and flagellin-toxin proteins expressed in *E. coli* BL21-Gold(DE3). (a) Samples of extracellular, soluble and insoluble protein fractions, as indicated in the figure, were resolved by SDS-PAGE. (b) Proteins from a duplicate, unstained gel were electroblotted onto a polyvinylidene fluoride (PVDF) membrane and subjected to Western blot analysis with an anti-epsilon antibody. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figure.

2.3.2.2 Cellular localization of the recombinant ETX0 protein

It is interesting to note that despite having cloned the full-length *etx* gene, which encodes for the epsilon prototoxin, the recombinant ETX0 protein banded at a position of 32 kDa in the SDS-polyacrylamide gel (Figs. 2.6a and 2.7). The epsilon prototoxin, however, is composed of 328 amino acids, inclusive of a 32-amino-acid signal peptide sequence, and has a predicted molecular mass of 36 kDa. A plausible explanation for the obtained results is therefore that the recombinant ETX0 protein might have been processed in *E. coli* so that the leader peptide sequence was removed, thus allowing for export of the mature protein from the bacterial cells. Such processing of the epsilon prototoxin would result in a mature epsilon toxin with a predicted molecular mass of 32 kDa, which is in agreement with that of the recombinant ETX0 toxin observed in the SDS-polyacrylamide gels. Conversely, due to the deletion of nine amino acids from the signal peptide sequence in the recombinant ETX1protein, this truncated toxin might not be processed and would therefore be expected to accumulate intracellularly. Supporting evidence for this assertion can be derived from the observation that the recombinant ETX0 protein could be detected in both the extracellular and intracellular protein fractions, albeit that the protein was present mostly in the soluble cytoplasmic fraction (Fig. 2.6a). In contrast, the recombinant ETX1 protein could be detected only in the insoluble cytoplasmic fraction, whereas the recombinant NC-ETX1 protein was also detected predominantly in the insoluble cytoplasmic protein fraction (Figs. 2.6b and 2.6c, respectively). Notably, the recombinant ETX1 and NC-ETX1 proteins, which both contain the identical aberrant signal peptide sequence, could not be detected in the extracellular protein fractions.

Based on the above, it was of interest to determine whether the recombinant ETX0 protein is indeed translocated to the *E. coli* periplasm and then secreted to the extracellular medium. To investigate, *E. coli* BL21-Gold(DE3) cells transformed with pEtx0 was cultured in EnPresso™ medium and the cells were harvested at 2 h post-induction to minimize cell lysis. The cell-free culture supernatant (extracellular protein fraction) was retained, and the cells were fractionated into cytoplasmic and periplasmic protein fractions. For the latter, an osmotic shock protocol was followed that allows for release of periplasmic proteins without concomitant cell lysis (Ausubel *et al.*, 1989). The protein samples were subsequently analyzed on an SDS-polyacrylamide gel. The results, presented in Fig. 2.8a, indicated that the recombinant ETX0 protein was mainly sequestered in the cytoplasmic fraction, but the

expressed protein also accumulated in the periplasmic space of *E. coli*. This suggested that the recombinant ETX0 protein was produced with a signal peptide sequence that allowed it to cross the cytoplasmic membrane.

Although the presence of recombinant ETX0 protein in the culture supernatant was suggestive of the possibility that the expressed protein was secreted, the possibility that cell lysis may have been responsible for its extracellular localization could not be excluded. To distinguish between these two possibilities, the cytoplasmic and extracellular protein fractions were subjected to Western blot analysis with an anti-GroEL monoclonal antibody. The GroEL protein is located in the cytoplasm of cells and is responsible for promoting the refolding and proper assembly of unfolded or misfolded polypeptides (Zeilstra-Ryalls *et al.*, 1991; Paal *et al.*, 2009). For comparative purposes, identically prepared protein fractions of *E. coli* BL21-Gold(DE3) cells transformed with the parental pET-28a(+) vector DNA were also included in the assay. Analysis of the Western blot (Fig. 2.8b) indicated that the cytoplasmic GroEL protein was detected in the extracellular medium of *E. coli* cultures harbouring the recombinant and non-recombinant bacterial expression vector. This suggested that cell lysis, and not active export, could be responsible for the observed presence of the recombinant ETX0 protein in the culture supernatant.

2.3.3 Optimization of recombinant protein expression in *E. coli* BL21-Gold(DE3)

Previously it was shown that the recombinant epsilon toxins and flagellin-toxin fusion proteins were expressed successfully in *E. coli* BL21-Gold(DE3) (Fig. 2.6). To facilitate future purification of these recombinant proteins it was necessary to optimize the yield of expressed recombinant proteins. For this purpose, different cultivation media and concentrations of the IPTG inducer were evaluated in an attempt to not only increase the yield of recombinant protein, but also to maximize expression of soluble products. The media evaluated in this study were LB medium, which is typically used for the production of heterologous proteins in *E. coli* (Sambrook and Russel, 2001), and EnPresso™ medium, which has been developed to provide an optimal cultivation environment for *E. coli* to reach a high cell density and protein product yields (BioSilta Product Information Sheet; Krause *et al.*, 2010). Several reports have also indicated that the level of expression and the solubility of the heterologously expressed protein can be improved by adjusting the concentration of IPTG

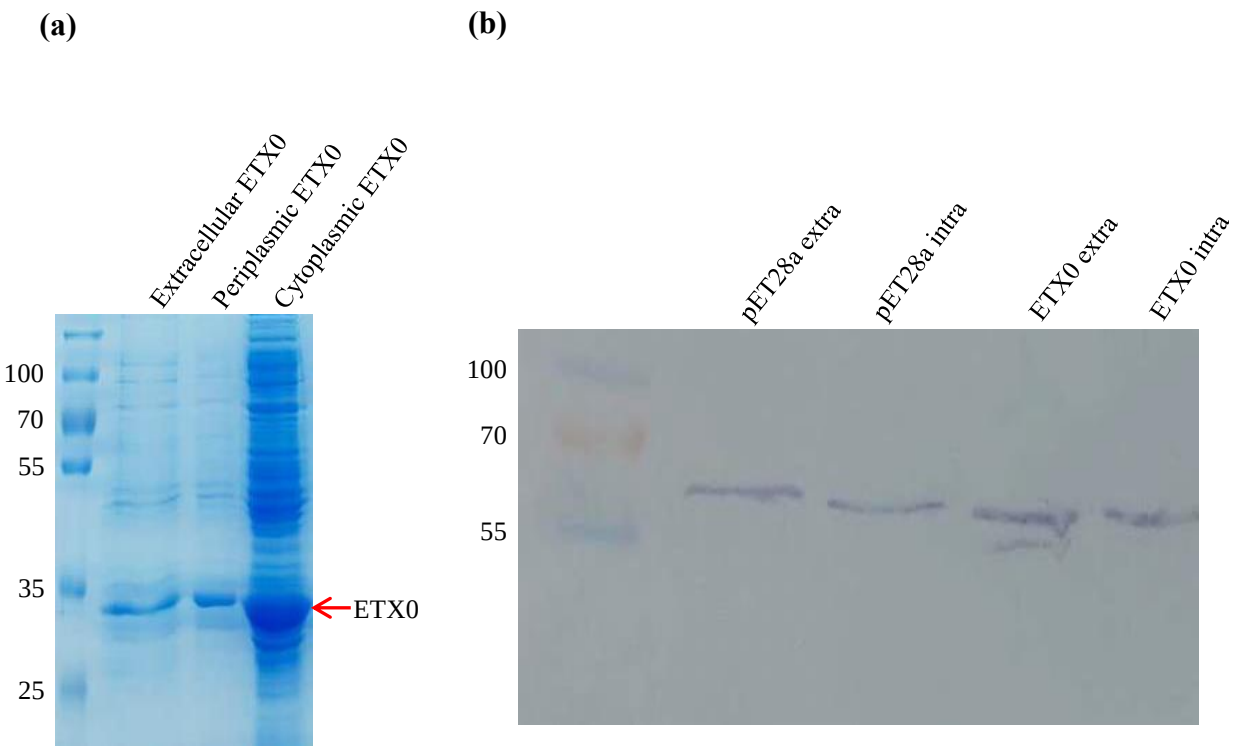


Fig. 2.8 Cellular localization of the recombinant ETX0 protein. (a) An IPTG-induced culture of *E. coli* BL23-Gold(DE3) cells transformed with recombinant plasmid pEtx0 was harvested at 2 h post-induction. The cells were fractionated and samples of the extracellular protein fraction, as well as periplasmic and cytoplasmic protein fractions were resolved by 12% SDS-PAGE. The position of the recombinant ETX0 protein is indicated with an arrow. (b) Samples of the extracellular (extra) and cytoplasmic (intra) protein fractions were subjected to Western blot analysis with an anti-GroEL antibody. In this experiment, identically prepared protein fractions from IPTG-induced *E. coli* BL23-Gold(DE3) cells transformed with the non-recombinant pET-28a(+) plasmid were included for comparative purposes. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figures.

(Kopetzki et al., 1989; Donovan et al., 1996; Sadeghi et al., 2011), thus making it an attractive parameter for optimization.

2.3.3.1 Recombinant protein expression in different cultivation media

To evaluate different cultivation media for optimal expression of the recombinant proteins, the expression levels of the recombinant ETX0 protein was determined in LB and EnPresso™ media. Cultures of *E. coli* BL21-Gold(DE3) cells harbouring the recombinant pEtx0 plasmid were inoculated into the respective media, induced with 1 mM IPTG and incubated at 30°C. The LB broth culture was incubated for 20 h, whereas the EnPresso culture was incubated for 24 h. Following incubation, total protein samples were analyzed by SDS-PAGE in the presence of the extracellular, as well as soluble and insoluble cytoplasmic protein samples. The recombinant ETX0 protein in each sample was quantified densitometrically using Gene Tools (SynGene) software.

Optical density measurements of the respective bacterial cultures at the end of the induction period indicated that the EnPresso™ medium was superior to the LB broth with regards to the cell density achieved. The final OD_{600} (at 24 h) in the EnPresso cultures was 9-14 and the final OD_{600} (at 20 h) in LB cultures was 3-4. Therefore, it is not surprising that the highest yield of the recombinant ETX0 protein was obtained when the bacteria were cultivated in EnPresso™ medium. After the induction period the total yield of recombinant ETX0 protein in the EnPresso culture was determined to be 302 mg/l, whereas the total yield in the LB culture was 179 mg/l (Fig. 2.9). In both the EnPresso™and LB cultivation media, the recombinant ETX0 protein was mainly expressed in a soluble form and represented 66.3% and 57.5%, respectively, of the total protein concentration. However, in contrast to the LB culture, the increased production of soluble ETX0 in the EnPresso culture appeared to coincide with a marked reduction in the yield of insoluble protein. In the EnPresso culture there was 7.5-fold more soluble protein compared to insoluble protein, but in the LB culture this difference was only 2-fold (Fig. 2.9).

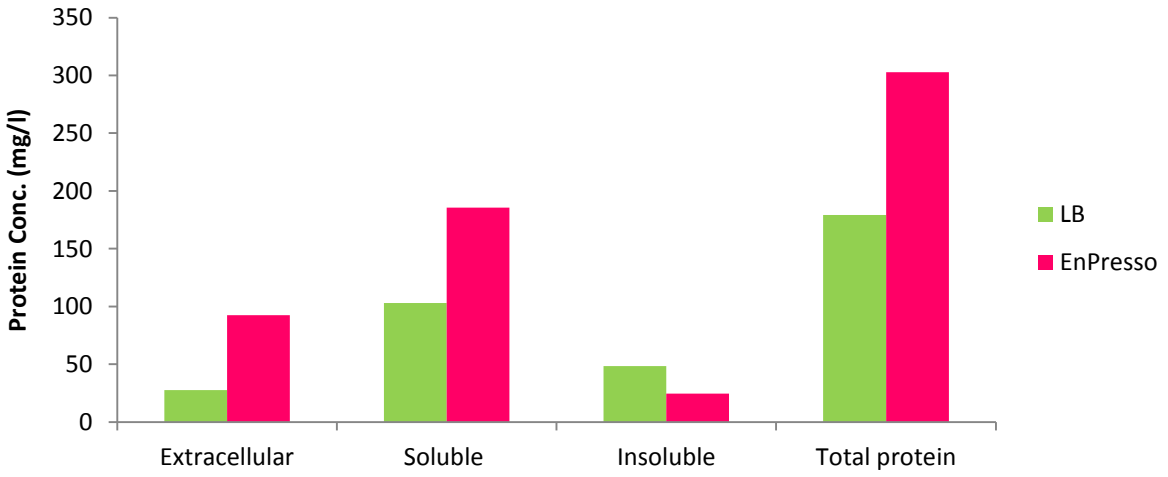


Fig. 2.9 Recombinant ETX0 protein production in *E. coli* BL21-Gold(DE3) cells using different cultivation media. The bacterial culture was cultivated in LB or EnPresso™ medium and induced with 1 mM IPTG. The LB culture was harvested at 20 h post-induction and the EnPresso culture at 24 h post-induction. Samples of the total protein, extracellular, as well as soluble and insoluble cytoplasmic proteins were subjected to SDS-PAGE and the yield of ETX0 in each protein sample was quantified by densitometry. The results are presented as the average of three assays performed for LB cultures, and the average of four assays performed for EnPresso cultures.

2.3.3.2 Optimization of the IPTG inducer concentration

The EnPresso™ medium was selected for use due to the high cell density obtained and the many fold increase in the yield of recombinant protein, especially soluble ETX0, as compared to the use of LB medium. Subsequently, different concentrations of the IPTG inducer were evaluated as a means to further optimize the overexpression of the recombinant proteins. In these experiments the *E. coli* BL21-Gold(DE3) strains harbouring recombinant plasmids pEtx0, pEtx1 or pNC-Etx1 were cultivated in EnPresso® medium and induced with IPTG concentrations ranging between 0.1 and 0.75 mM. Following the induction period, protein samples were prepared and the concentration of the recombinant proteins in each protein fraction was quantified by densitometry, as highlighted above. The results of these assays are presented in Fig. 2.10.

The highest yields of recombinant and soluble ETX0 (828 mg/l and 744 mg/l, respectively) were obtained when the bacterial culture was induced with 0.1 mM IPTG. A steady decline in the yield of recombinant ETX0 protein was observed as the concentration of IPTG was increased, and the yield of the recombinant protein in cultures induced with 0.75 mM IPTG was 1.6-fold less than that obtained in cultures induced with 0.1 mM IPTG (Fig. 2.10a). Notably, the total yield of recombinant ETX0 protein and the yield of soluble protein was respectively 2.7-fold and 4-fold higher than the yields obtained above in cultures induced with 1 mM IPTG.

In agreement with previous results (Fig. 2.6b), the recombinant ETX1 protein was expressed in an insoluble form and the yield of soluble protein was negligible, irrespective of the concentration of IPTG used. The highest yield of ETX1 (395 mg/l) was obtained when the bacterial culture was induced with 0.75 mM IPTG, and similar yields were obtained in bacterial cultures induced with 0.5 or 0.25 mM IPTG (365 mg/l and 384 mg/l, respectively). The yield of the recombinant ETX1 protein was 2.9-fold less in bacterial cultures induced with 0.1 mM IPTG compared to those induced with 0.75 mM IPTG (Fig. 2.10b).

The recombinant NC-ETX1 protein was produced in a predominantly insoluble form, irrespective of the concentration of IPTG used. Nevertheless, the highest yield of NC-ETX1 (525 mg/l) was obtained when the bacterial culture was induced with 0.1 mM IPTG and at this concentration of inducer the yield of soluble protein was also the highest (98 mg/l).

Comparable yields of the recombinant NC-ETX1 protein was obtained in bacterial cultures induced with higher concentrations of IPTG and ranged from 462 mg/l for cultures induced with 0.5 or 0.75 mM IPTG to 403 mg/l for cultures induced with 0.25 mM IPTG (Fig. 2.10c).

Cumulatively, the results indicated that optimal production of the different recombinant proteins in *E. coli* BL21-Gold(DE3) can be achieved through cultivating the bacterial strains in EnPresso™ medium and inducing the bacterial cultures with 0.1 mM IPTG (ETX0 and NC-ETX) or 075 mM IPTG (ETX1) for a period of 24 h at 30°C.

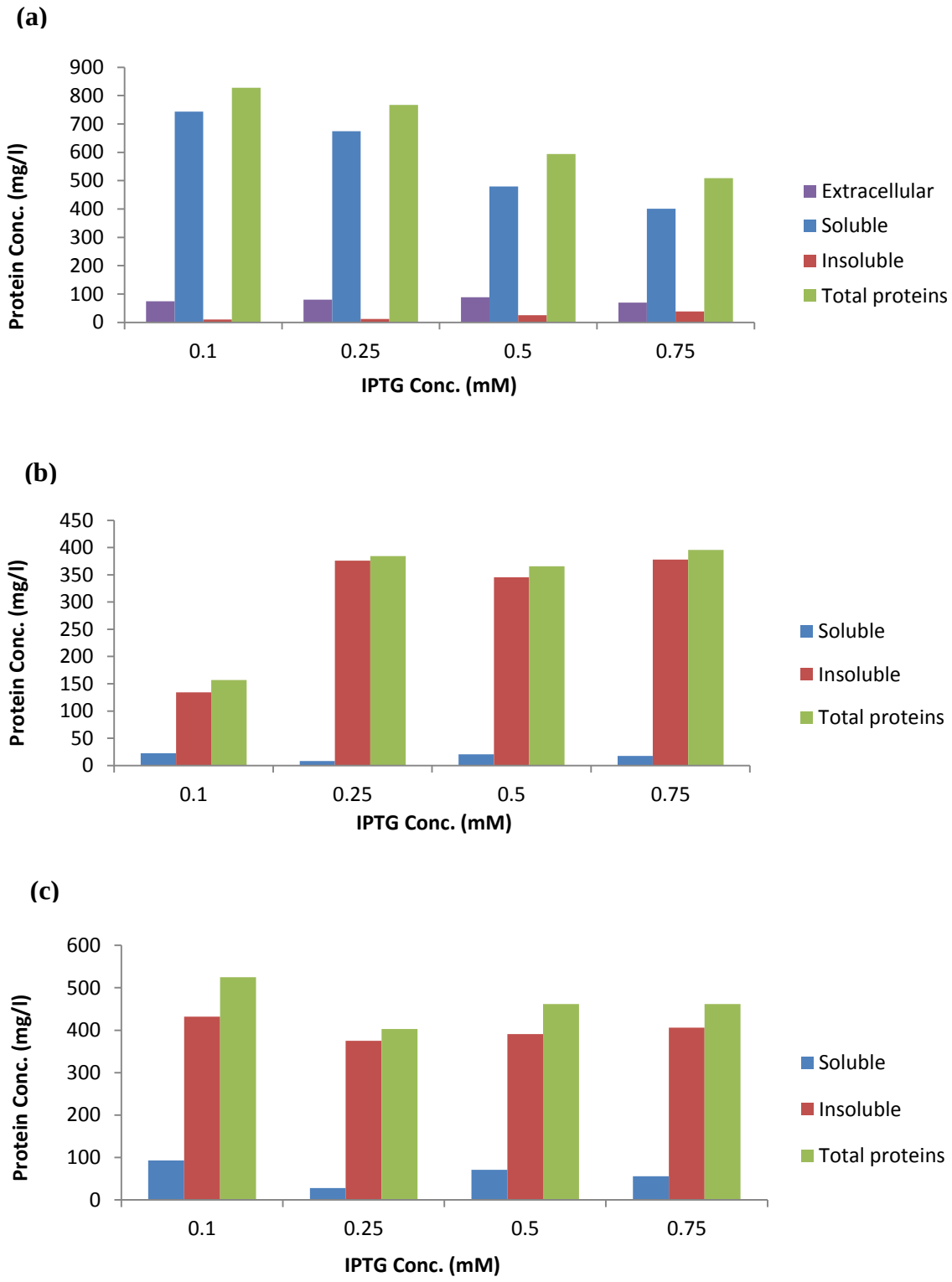


Fig. 2.10 Recombinant protein production in *E. coli* BL21-Gold(DE3) cells using different concentrations of the inducer. Bacterial cultures expressing the recombinant ETX0 (a), ETX1 (b) and NC-ETX1 (c) proteins were cultivated in EnPresso™ medium and induced with different concentrations of IPTG that ranged between 0.1 and 0.75 mM. Protein samples were prepared at 24 h post-induction and the yield of the respective recombinant proteins were quantified as described in the legend to Fig. 2.9. The results are presented as the average of three assays performed on the cultures. Since the recombinant ETX1 and NC-ETX1 proteins were not detected in the extracellular protein fraction, these samples were excluded from the analyses.

2.4 DISCUSSION

Over the past 15 years, the variety of hosts and vector systems for recombinant protein expression has increased dramatically. It is now possible to select from mammalian (Lundstrom, 2003), insect (Kost and Condreay, 1999; Jarvis, 2009), yeast (Daly and Hearn 2005) and prokaryotic (Baneyx, 1999; Schumann and Ferreira, 2004; Schumann, 2007) hosts and expression vector systems. Despite these developments, *E. coli* has remained one of the most attractive hosts for heterologous protein production, because of its ability to grow rapidly and at a high density on inexpensive substrates, its well characterized genetics, and the availability of a large number of expression vectors and mutant host strains (Baneyx, 1999; Sørensen and Mortensen, 2005; Samuelson, 2011). Consequently, the aims of this part of the study were essentially to express the C. *perfringens* Type D epsilon toxin and a flagellin-toxin fusion protein in *E. coli*, and to optimize the expression of these recombinant proteins for future use as potential vaccine candidates against enterotoxemia.

A factor complicating high-level expression of the Type D epsilon toxin in heterologous hosts is the low G+C content (27%) of the structural gene encoding for the toxin (Garnier and Cole, 1986; Hunter *et al.*, 1992). This may point to differences in codon usage and thus a codon bias. In bacteria, the level of tRNA appears to be directly proportional to the frequency of codon usage (Kane, 1995). Ikemura (1981) noted that the most highly expressed genes in *E. coli* contain mostly codons corresponding to major tRNAs, but few codons of minor tRNAs. In contrast, genes that were expressed less well use more suboptimal codons. In addition to affecting the level of expression, a shortage of charged tRNA during protein synthesis can lead to misincorporation of amino acids and frame-shifting (McPherson, 1988). Thus, the accuracy of translation, as opposed to its rate, may also be affected by codon choice. Several reports have indicated that ribosomes stalled at rare codons can frameshift, hop or terminate, thereby affecting both the level of expression and quality of heterologous protein (Gallant and Lindsley, 1998; Kane, 1995; Buchan *et al.*, 2006). It therefore follows that for optimal expression of the epsilon toxin in heterologous hosts, it may be advisable to rather optimize the codon choice in accordance with that used by the translational machinery of the expression host. Indeed, by making use of an epsilon gene that was generated by overlapping PCR to contain codons favourable for high-level expression in *E. coli*, it was reported that expression of the recombinant protein reached 35% of the total protein concentration (Zhao *et al.*, 2011).

Based on the above considerations, a synthetic *E. coli* codon-optimized gene was used as the source for the expression of the proteins reported in this study. Genes encoding the full-length (*etx*0) and truncated (*etx*1) epsilon toxin, as well as a flagellin-toxin (Δ*hag::etx*1) fusion protein were cloned into the bacterial expression vector pET-28a(+). After induction with IPTG, protein samples of the derived recombinant *E. coli* strains were analyzed by SDS-PAGE. The respective recombinant proteins were found to be expressed and their identity was subsequently confirmed by Western blot analysis using an anti-epsilon toxin antibody.

In C. *perfringens* the epsilon toxin is expressed as an inactive prototoxin that is preceded by a signal peptide, resulting in the mature protein being exported from the bacteria (Bhown and Habeeb, 1977). In this study, the recombinant ETX0 protein was expressed in a soluble (correctly folded) form and could also be detected in the cell-free culture supernatant. The latter suggested that the recombinant ETX0 protein was secreted and, if indeed the case, may facilitate greatly the recovery and purification of the recombinant protein. To investigate this aspect in greater detail, the cellular localization of the ETX0 protein was determined. Similar to results noted by Hunter *et al.* (1992) and Oyston *et al.* (1998), the results of these investigations indicated that the toxin accumulated in the periplasmic space of *E. coli*, albeit to noticeably lower levels compared to that observed in the cell cytoplasm. These results suggested that the recombinant ETX0 protein was produced with a signal peptide that allowed it to cross the cytoplasmic membrane. However, it is possible that due to the high level of recombinant protein production, the translocation machinery of *E. coli* is overwhelmed and thus resulted in the cytoplasmic accumulation of the protein (Talmadge and Gilbert, 1982; Wilcox and Studnicka, 1988; Gottesman, 1996). In this study, active export from the periplasm to the extracellular milieu could not be demonstrated and the presence of recombinant ETX0 protein in the culture supernatant was shown to be most likely due to cell lysis.

In contrast to ETX0, the recombinant ETX1 and NC-ETX1 proteins were expressed in an insoluble form. The accumulation of heterologous proteins into inclusion bodies is a frequent occurrence in *E. coli* (Villaverde and Carrio, 2003; Sørensen and Mortensen, 2005) and these aggregated insoluble proteins generally represent misfolded proteins (Mitraki and King, 1989; Thomas and Baneyx, 1996; Villaverde and Carrio, 2003). Under normal cellular conditions a subset of cytoplasmic proteins are able to fold spontaneously (Anfinsen, 1973), while aggregation-prone proteins require the assistance of a number of molecular chaperones

that interact reversibly with nascent polypeptide chains to prevent aggregation during the folding process (Hartl and Hayer-Hartl, 2002). Aggregation of the recombinant ETX1 and NC-ETX1 proteins over-expressed in *E. coli* cells could therefore have resulted either from accumulation of high concentrations of folding intermediates or from inefficient processing by molecular chaperones. The precise physiochemical parameters that contribute to the formation of inclusion bodies remain unclear (Krueger *et al.*, 1989; Rudolph and Lilie, 1996; Sørensen and Mortensen, 2005), but properties of the expressed target protein such as charge average, turn-forming residue fraction, cysteine and proline fractions, hydrophobicity, and total number of residues may all aid in inclusion body formation (Wilkinson and Harrison, 1991). However, the accumulation of the ETX1 and NC-ETX1 proteins in inclusion bodies may not necessarily be undesirable, since inclusion bodies can represent the highest yielding fraction of target protein, they are easy to isolate and target proteins in inclusion bodies are generally protected from proteolytic breakdown (Mukhopadhyay, 1997; Villaverde and Carrio, 2003).

Several approaches to limit the *in vivo* aggregation of recombinant proteins have been described. A well-known approach consists of cultivation at reduced temperatures (Cabilly, 1989; Schein, 1993; Vasina and Baneyx, 1996a). The aggregation reaction is generally favoured at higher temperatures due to the strong temperature dependence of hydrophobic interactions that determine the aggregation reactions (Kiefhaber *et al.*, 1991). A direct consequence of temperature reduction is the partial elimination of heat shock proteases that are induced under over-expression conditions. Furthermore, the activity and expression of a number of *E. coli* chaperones are increased at temperatures around 30°C (Mogk *et al.*, 2002; Ferrer *et al.*, 2003). Consequently, the cultures used in this study were grown at 30°C after induction with IPTG. However, despite the potential for correct folding at this lower temperature, the recombinant ETX1 and NC-ETX1 proteins were still found to be insoluble.

Another important factor in expression of soluble recombinant proteins is medium composition and pH (Sugimoto *et al.*, 1991; Weickert *et al.*, 1996; Lewis *et al.*, 2004; Krause *et al.*, 2010). In this study, both LB and EnPresso™ media were evaluated for high-level expression of the recombinant ETX0 protein. The results indicated that the EnPresso™ medium was superior to the LB medium since not only were higher cell densities achieved, but the yield of recombinant protein was 1.7-fold higher and the proportion of soluble proteins in the harvested cells were also 1.8-fold higher (Fig. 2.9). These differences may be

directly ascribed to the differences in the cultivation media used. In the case of LB cultures, the culture components are all added in the flask at the start of the cultivation. Consequently, high cell densities cannot be reached since the high respiratory rate of fast growing bacteria exceeds the oxygen transfer capacity of the cultivation vessel, and the culture therefore soon becomes depleted of oxygen. Under oxygen limitation, growth is slow and recombinant protein production is poor (Vasala *et al.*, 2006; Krause *et al.*, 2010). Moreover, pH maintenance is hampered by metabolic events such as utilization of complex compounds (e.g. yeast extract, protein peptones, casamino acids) as a carbon source, which results in an increase in the pH due to excess ammonia being secreted into the medium (Warnecke and Gill, 2005). In contrast, the EnPresso™ culture medium combines an enzymatic glucose release system with an optimized medium (mixture of mineral salts and complex additives) that allows for controlled cell growth. Therefore, accumulation of harmful metabolites can be minimized and appearance of anaerobic conditions can be avoided. Furthermore, the addition of a high dose of complex nutrients ("booster") at the time of induction not only provides a sufficient supply of the key medium compounds (e.g. amino acids, trace metals, co-factors and vitamins) needed for efficient protein synthesis, but it also helps to maintain the pH at 7.0 (Krause *et al.*, 2010).

Because the characteristics of each target gene to be expressed are unique, the optimal concentration of transcriptional inducer can vary (Donovan *et al.*, 1996; Malakar and Venkatesh, 2011). In contrast to the use of a strong promoter such as the T7 RNA polymerase promoter in the pET-28a(+) vector, moderately strong or weak promoters may favour correct folding of recombinant proteins due to the reduction in cellular protein concentration (Giladi *et al.*, 1995). The same result may, however, be obtained by partially inducing strong promoters through using different concentrations of the IPTG inducer (Weickert *et al.*, 1996; Donovan *et al.*, 1996; Malakar and Venkatesh, 2011). Among the different IPTG concentrations evaluated in this study (0.1 to 0.75 mM), an IPTG concentration of 0.1 mM was shown to yield the highest levels of protein expression and the highest amounts of soluble protein for both the ETX0 and NC-ETX1 proteins. In contrast, the highest yield of the recombinant ETX1 protein was obtained when the bacterial culture was induced with 0.75 mM IPTG, and varying the concentration of inducer did not enhance the production of soluble protein.

Using the optimized conditions for recombinant protein expression (EnPresso™ medium,

IPTG concentration of 0.1 mM or 0.75 mM, induction at 30°C for 24 h), the yields of ETX0, ETX1 and NC-ETX1 were determined to be 828 mg/l, 395 mg/l and 525 mg/l, respectively. These results compare favourably to published literature for which the yield of recombinant epsilon toxins have been provided. Previous studies regarding expression of the epsilon prototoxin indicated that the protein was expressed in a soluble form, albeit to very low levels that ranged between 5-25 mg/l (Hunter *et al.*, 1992). In contrast, expression of the full-length Type D epsilon toxin in *E. coli* using pQE bacterial expression vectors were reported to result in the expression of insoluble protein and the total yield was reported to be 20 mg/l (Goswami *et al.*, 1996) and 10-12 mg/l (Chandran *et al.*, 2010), respectively. Compared to the results obtained in this study, the yield of the recombinant ETX0 protein was at least 33-fold higher than that reported by Hunter *et al.* (1992), 41-fold higher than that reported by Goswami *et al.* (1996) and at least 69-fold higher than that reported by Chandran *et al.* (2010). Similar to the results obtained in this study, previous studies have indicated that the truncated epsilon toxin of C. *perfringens* Type D was insoluble when expressed in *E. coli* using pET-11a expression vectors and yields of between 10-15 mg/l were reported (Lobato *et al.*, 2010; Souza *et al.*, 2010). However, in contrast to these studies, the yield of recombinant ETX1 reported here was ca. 26- to 39-fold higher than that reported previously.

In conclusion, the epsilon toxin of *C. perfringens* Type D was successfully over-expressed as a holotoxin, an N-terminal truncated toxin and as flagellin-toxin fusion protein in *E. coli.* In contrast to the recombinant ETX0 protein, the ETX1 and NC-ETX1 proteins were found to be insoluble. Despite the use of different approaches aimed at allowing expression of soluble recombinant protein *in vivo*, none of these resulted in enhanced expression of soluble ETX1 and NC-ETX1. Nevertheless, it is possible to purify insoluble recombinant proteins by denaturing the proteins followed by refolding into their native conformation. The details of these types of studies are provided in the following Chapter.

CHAPTER 3

Purification of recombinant *Clostridium perfringens* Type D epsilon toxins and a flagellin-toxin fusion protein

3.1 INTRODUCTION

Vaccination is the most efficient prophylaxis against a variety of infectious diseases (Meeusen *et al.*, 2007; Rueckert and Guzmán, 2012). In the case of veterinary vaccines, the majority of licensed vaccines comprise inactivated or attenuated microorganisms or toxoids (Rogan and Babiuk, 2005; Meeusen *et al.*, 2007; Plotkin and Plotkin, 2011). Inactivated vaccines, which result from the inactivation or killing of the pathogens by chemical or physical means, are non-replicating and therefore considered to be safe. However, these vaccines are expensive to produce due to additional downstream processing requirements that include formulation with an adjuvant or immuno-stimulant and the need for a high antigen payload in the vaccine dose (van Oirschot, 1997). On the other hand, live attenuated vaccines are derived from either mutant strains or artificially attenuated pathogenic microorganisms that have reduced virulence in the target host, while maintaining immunogenicity. Despite being capable of generating long-lasting immunity in vaccinated animals, live attenuated vaccines carry the potential risk of residual virulence and the potential for release of the new organisms into new environments (Ferrari *et al.*, 2005; Batten *et al.*, 2008). Toxoid vaccines are based on toxins secreted from an organism, which are altered in such a way as to reduce their inherent levels of toxicity, usually through chemical treatment with formaldehyde (Walker, 1992). Since bacterial toxins are not produced in significant quantities in synthetic media, they typically require a source of peptides, most of which are natural products such as meat, casein or soybean (Bhown and Habeeb, 1977; Walker, 1997; Goncalves *et al.*, 2009). The use of meat-based products in the cultivation medium has become problematic since the emergence of bovine spongiform encephalitis, thus complicating the registration and acceptability of some toxoid vaccines (Cashman, 2001).

Over the past two decades, there has been increasing pressure applied by regulatory authorities, both human and veterinary, to specifically define the protective antigens and produce vaccines that would be free from pathogen-associated toxins, extraneous agents and immunosuppressive components (Castle, 2005; Gifford *et al.*, 2011). Recombinant subunit vaccines, based on the production of recombinant protein antigens, are currently the most cost-effective method of producing antigens free from extraneous material that is characteristic of the above-mentioned conventional vaccines (Hansson *et al.*, 2000; Rogan and Babiuk, 2005). Initially, subunit vaccines were produced by purifying the specific antigens from cultures of the pathogenic bacteria. Besides being expensive due to the large-

scale production required, this approach has the potential risk of generating an antigen that may still contain residues of unwanted material or virulence. However, the advent of molecular biology and genetic engineering has impacted greatly on vaccine development by providing the tools and techniques necessary to produce a single protein in a suitable expression system. As a consequence, several recombinant subunit vaccines are available commercially, i.e. vaccines against feline leukaemia virus (Marciani *et al.*, 1991), Lyme disease (Chang *et al.*, 1995) and classical swine fever (Rau *et al.*, 2006). Since these recombinant subunit vaccines are composed of a purified protein, they are characterized by increased safety profiles, potency and harbour less reactogenic substances (Hansson *et al.*, 2000; Unnikrishnan *et al.*, 2012).

Clostridium perfringens Type D infection results in enterotoxemia of livestock animals and is responsible for widespread mortality, resulting in heavy economic losses worldwide (Songer, 1996; Uzal, 2004). The death of the animal may occur within 2 h after the onset of clinical signs. Due to the rapid rate of progression of the disease, prevention of the disease is essential. The currently available vaccines are based on formaldehyde-treated whole-cell cultures or bacterial culture filtrates (Titball, 2009). Although these vaccines confer a good degree of protection in animals, they contain a biological mixture of uncharacterized antigens from the bacterium and may also contain toxic by-products in addition to the epsilon toxoid (Bhown and Habeeb, 1977). As a consequence, the immunogenicity of the preparations is variable from batch to batch (Uzal and Kelly, 1998) and the vaccine is known to fail sometimes for reasons of potency (Titball, 2009). In addition, inflammatory responses following vaccination have also been reported (Stokka *et al.*, 1994).

Towards generating a safe and reliable vaccine that can be conveniently produced in large amounts, the *C. perfringens* Type D epsilon toxin (ETX0) and modified versions thereof (ETX1 and NC-ETX1) have been expressed to high levels in *Escherichia coli* (Chapter 2). These proteins were expressed with a N-terminal hexahistidine tag in order to facilitate their purification. Consequently, the aims of this part of the study were (i) to purify the recombinant ETX0, ETX1 and NC-ETX1 proteins, and (ii) to evaluate their cytotoxicity *in vitro* against Madin-Darby canine kidney (MDCK) cells.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

Madin-Darby canine kidney (MDCK) cells, kindly provided by Onderstepoort Biological Products, Pretoria, were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 100 U/ml of penicillin G and 100 µg of streptomycin sulfate (Gibco). The cell culture medium was supplemented with 10% (v/v) fetal bovine serum (Gibco) and the cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

3.2.2 Bacterial strains and culture conditions

The bacterial strains used in this part of the study are listed in Table 3.1. The *E. coli* strains were cultivated in Luria-Bertani (LB) broth (0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7) at 37°C with shaking at 200 rpm, and maintained -70°C as glycerol cultures. For recombinant protein expression, *E. coli* strains were induced and cultivated in EnPresso™ medium (BioSilta), which was prepared according to the manufacturer's specifications. For plasmid DNA maintenance in *E. coli*, 50 µg/ml of kanamycin (Sigma-Aldrich) was added to the cultivation media.

Table 3.1 Bacterial strains used in this study

Strains	Characteristics	Source or Reference
E. coli BL21-Gold(DE3)	Expression host; F^- *ampT hsdS* (r_B- m_B-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte	Novagen
E. coli BL21(DE3)[pEtx0]	*E. coli* BL21-Gold(DE3) transformed with pET-28a(+) harbouring a synthetic codon-optimized *C. perfringens* Type D *etx* gene	This study; Chapter 2
E. coli BL21(DE3)[pEtx1]	*E. coli* BL21-Gold(DE3) transformed with pET-28a(+) harbouring a truncated *etx*1 gene that lacks the first nine N-terminal amino acids	This study; Chapter 2
E. coli BL21(DE3)[pNCetx1]	*E. coli* BL21-Gold(DE3) transformed with pET-28a(+) harbouring a fusion gene between the truncated Δ*hag* and *etx*1 genes	This study; Chapter 2

3.2.3 Recombinant protein expression in *E. coli*

Pre-cultures of the recombinant *E. coli* BL21-Gold(DE3) strains were prepared by inoculating 5 ml of LB broth with 100 µl of the glycerol bacterial culture. Following incubation at 37°C for 7 h with shaking at 200 rpm, 500 µl of the bacterial culture was transferred to 50 ml of EnPresso™ broth in 500-ml baffled flasks and 0.3 U of EnZ I'm (BioSilta) was added to the flask. The flask was sealed with Steri-Wrap® and incubated at 37°C for 16 h with shaking. After incubation, the culture was supplemented with a booster tablet, supplied with the medium, and 0.6 U of EnZ I'm. The culture was simultaneously induced for recombinant protein expression by addition of 0.1 mM IPTG (for ETX0 and NC-ETX) or 0.75 mM IPTG (for ETX1) and then incubated at 30°C for 24 h with shaking at 200 rpm.

3.2.4 Small-scale purification of recombinant proteins

The recombinant hexahistidine-tagged ETX0, ETX1 and NC-ETX1 proteins were purified by immobilized metal affinity chromatography (IMAC) using the MagneHis™ Protein Purification System (Promega) according to the manufacturer's instructions. For this purpose, the ETX0 protein was purified from the extracellular, periplasmic and soluble cytoplasmic protein fractions, whereas the insoluble ETX1 and NC-ETX1 proteins were solubilized in 8 M urea prior to purification. The cells from 1 ml of induced cultures of *E. coli* BL21(DE3)[pEtx0], *E. coli* BL21(DE3)[pEtx1] and *E. coli* BL21(DE3)[pNCetx1] were pelleted by centrifugation at 7000 × *g* for 2 min and suspended in 270 µl of distilled water. To lyse the bacterial cells, 30 µl of the supplied FastBreak™ Reagent and 1 µl of DNase I solution was added. Following incubation for 20 min at room temperature with shaking (50 rpm), NaCl was added to a final concentration of 500 mM and then 60 µl of the MagneHis™ paramagnetic nickel-particles was added with a wide-bore pipette tip. The contents of the tubes were mixed thoroughly and left to stand for 2 min at room temperature. The MagneHis™ nickel particles were subsequently captured with a DynaMag®-2 magnetic stand (Invitrogen), and the protein/particles were washed three times with 150 µl of MagneHis™ Binding/Wash buffer. The proteins were subsequently eluted with 100 µl of MagneHis™ Elution buffer (containing 500 mM imidazole). For purification of the recombinant ETX0 protein from the cell-free culture supernatant, an aliquot of the supernatant (1 ml) was added directly to 60 µl of the MagneHis™ resin and processed as described above.

The identity of the purified recombinant proteins was confirmed by Western blot analysis as described previously (Chapter 2, Section 2.2.12.2), except that an anti-polyhistidine monoclonal antibody conjugated to horseradish peroxidase (Sigma-Aldrich) was used at a dilution of 1:2000.

3.2.5 Ion exchange chromatography

Large-scale purification of the recombinant ETX0 protein was performed through ion exchange chromatography by making use of the strong anion exchanger resin, Capto-Q (GE HealthCare). The cells from 50 ml of an induced culture of *E. coli* BL21(DE3)[pEtx0] were harvested by centrifugation at 7000 × *g* for 10 min and suspended in 50 ml of 0.5 M diethanolamine (DEA) buffer (pH 9.5). The cells were lysed by sonication on ice at 5-min intervals for 15 min with a Bendelin Sonopuls at a power output of 67%. The lysate was clarified by centrifugation at 16 000 × *g* for 20 min at 4°C. The supernatant was recovered and the pH was adjusted to 9.5. A XK 16/20 glass column (GE HealthCare) was packed with 25 ml of the Capto-Q resin and then equilibrated with three column volumes (CV; 75 ml) of 0.5 M DEA (pH 9.5), followed by loading of the protein sample directly onto the resin. The resin was washed with three CV of 0.5 M DEA (pH 9.5), after which the bound proteins were eluted with a linear gradient of 1 mM to 1 M NaCl in the equilibration buffer. Fractions were collected from the column into centrifuge tubes and aliquots were analyzed by 12% SDS-PAGE. Fractions containing the recombinant ETX0 protein were pooled and dialysis was performed to exchange the DEA buffer with PBS buffer (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl; pH 7.4). The dialysis was performed against 4 l of PBS buffer for 16 h in a cold room using SnakeSkin™ dialysis tubing (Pierce) with a 10 000 Dalton molecular weight cut-off limit.

3.2.6 Nickel affinity chromatography

The insoluble recombinant ETX1 and NC-ETX1 proteins were purified at a large scale from inclusion bodies by making use of a nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) and a Protino® nickel-tris(carboxymethyl)ethylene diamine (Ni-TED) resin (Macherey-Nagel).

3.2.6.1 Purification of NC-ETX1 with a Ni-NTA resin

- **Solubilization of the recombinant protein**

The cells from 50 ml of an induced culture of *E. coli* BL21(DE3)[pNCetx1] were harvested by centrifugation at 7000 × *g* for 10 min at 4°C. The bacterial cell pellet was suspended in 5 ml/g of the BugBuster™ protein extraction reagent (Novagen) and incubated on a shaking platform at 50 rpm for 20 min at room temperature. Following centrifugation at 6000 × *g* for 15 min, the pellet was washed with 50 ml of buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8) and centrifuged as before. The pellet was then suspended in 50 ml of denaturation buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 8) and incubated on a shaking platform at 50 rpm for 15 min at room temperature. Following incubation, the suspension was centrifuged at 16 000 × *g* for 20 min at 4°C and the supernatant, containing solubilized protein, was retained.

- **Affinity chromatography**

A XK16/20 glass column (GE HealthCare) was packed with 25 ml of Ni-NTA resin (Qiagen) and equilibrated with three column volumes (CV; 75 ml) of denaturation buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 8.0). The protein sample was loaded onto the resin, followed by washing with three CV of denaturation buffer, of which the pH was adjusted to 6.3 with 1 N HCl. To allow for refolding of the resin-bound proteins, a second wash step with three CV was performed using Buffer B (50 mM NaH_2PO_4, 300 mM NaCl; pH 8). The proteins were then eluted in Buffer B containing 250 mM imidazole. Fractions were collected from the column into centrifuge tubes and aliquots were analyzed by 12% SDS-PAGE.

3.2.6.2 Purification of ETX1 and NC-ETX1 with a Ni-TED resin

The insoluble recombinant ETX1 and NC-ETX1 proteins were also purified under denaturing conditions by employing a Protino® Ni-TED resin (Macherey-Nagel). Inclusion bodies of induced cultures of *E. coli* BL21(DE3)[pEtx1] and *E. coli* BL21(DE3)[pNCetx1] were purified with the BugBuster™ protein extraction reagent, as described above, except that the

inclusion bodies were solubilized in Protino® Denaturing Solubilisation (DS) buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea; pH 8). Following centrifugation at 16 000 × *g* for 20 min at 4ºC, the supernatant was recovered and used for purification of the recombinant proteins. For this purpose, a XK 26 glass column (GE HealthCare) was packed with 50 ml of the Protino® Ni-TED resin and was equilibrated with three column volumes (CV; 150 ml) of the DS buffer. The protein sample was loaded onto the resin, after which the resin was washed with three CV of DS buffer to remove unbound proteins. The proteins were subsequently eluted in Protino® Denaturing Elution (DE) buffer (50 mM NaH_2PO_4, 30 mM NaCl, 8 M urea, 250 mM imidazole; pH 8). Fractions were collected from the column into centrifuge tubes and aliquots were analyzed by 12% SDS-PAGE. Fractions containing the respective recombinant proteins were pooled and dialysis was performed to exchange the urea-containing DE buffer with TBS buffer (100 mM Tris, 100 mM NaCl; pH 10). As indicated previously, the dialysis was performed against 4 l of TBS buffer for 16 h in a cold room using SnakeSkin™ dialysis tubing (Pierce) with a 10 000 Dalton molecular weight cut-off limit.

3.2.7 Protein concentration determination

The concentration of purified recombinant proteins was determined by the method of Bradford (1976) with the Quick Start™ Protein Assay kit (BioRad), and with bovine serum albumin (BSA) as standard. Aliquots (20 µl) of each sample was mixed with 1 ml of Protein Assay reagent in disposable cuvettes, incubated at room temperature for 5 min and the absorbance at 595 nm was then determined with a Beckman Coulter DU® 800 spectrophotometer. Distilled water containing Protein Assay reagent was used to zero the absorbancy readings, and the protein concentration was determined from the prepared standard curve.

3.2.8 Activation of the recombinant ETX0 protein

Trypsin was used to convert the recombinant ETX0 protein, obtained from the cell-free culture supernatant, to an active toxin according to the method of Minami *et al.* (1997). For this purpose, 100 µl of the protein sample (ca. 40 µg of ETX0 protein) was added to 800 µl of PBS buffer, mixed thoroughly and then 100 µl of an $MgSO_4$/trypsin/EDTA solution (Biochrom) was added. The samples were incubated in a water bath at 37°C for 30 min.

Following incubation, aliquots of the protein samples were analyzed by Western blot analysis using a polyclonal anti-epsilon antibody as described previously (Chapter 2, Section 2.2.12.2).

3.2.9 Cytotoxicity assays

The toxicity of the recombinant ETX0, ETX1 and NC-ETX1 proteins towards Madin-Darby canine kidney (MDCK) cells was determined *in vitro* with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. The MDCK cells were seeded in 96-well microtitre plates with 100 µl of complete DMEM medium at a density of 1.5×10^4 cells/well. Following incubation for 24 h at 37°C in a 5% CO_2 incubator, the cell monolayers were rinsed once with DMEM and then incubated with serial dilutions of the respective recombinant proteins at a starting concentration of 100 µg/ml. The recombinant proteins were diluted in DMEM and 100 µl of each dilution was added per well. Following incubation for 24 h, 20 µl of a solution comprising of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) was pipetted into each well. Subsequently, the plates were incubated at 37°C in 5% CO_2 for 2 h. Following incubation, the absorbance at 490 nm was measured with a Tecan Infinite F500 ELISA plate reader and the IC_{50} values were determined. Untreated MDCK cells were included as a negative control in these assays, whereas cells treated with 100 µM Emetine were included as a positive control.

3.2.10 Protein identification by mass spectrometry

Regions of Coomassie blue-stained SDS-polyacrylamide gels containing protein bands of interest were excised. In-gel digestion of proteins and peptide extraction were performed, as described by Shevchenko *et al*. (2006). The samples were analyzed on an Agilent 1100 HPLC system equipped with capillary and nano LC pumps coupled to a QSTAR ELITE mass spectrometer. Aliquots (1-2 µg) of each sample were desalted on a Symmetry C18 trap column (0.18 × 23.5 mm) with Buffer 1 (0.5% acetonitrile [ACN], 0.5% formic acid [FA]). Peptides were separated on a NanoEase XBridge C18 column (0.1 × 50 mm) connected to the trap column via a six-port switching valve, and eluted in a gradient of Buffer 2 (98% ACN, 0.5% FA). Nano-spray was achieved using a MicroIonSpray head assembled with a New Objective PicoTip emitter and an electrospray voltage of 3 kV was applied to the emitter.

The mass spectrometer was operated in Information Dependant Acquisition (IDA) using an Exit Factor of 2.0 and Maximum Accumulation Time of 2.5 s. The MS scans were acquired from 400-1600 m/z and the three most intense ions were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Protein identification was performed using the Paraghon™ algorithm thorough Search in Protein Pilot (Protein Pilot v.3.0 software). An identification confidence of 95% was selected during searches.

3.3 RESULTS

3.3.1 Purification and characterization of recombinant proteins

It has previously been shown that the recombinant ETX0 protein was expressed in *E. coli* BL21-Gold(DE3) cells as soluble protein that accumulated in the cytoplasm of the cells, and to a lesser extent in the periplasm and culture supernatant. In contrast, the recombinant ETX1 and NC-ETX1 proteins were expressed in a predominantly insoluble form (Chapter 2, Figs. 2.7 and 2.8). Considering that these recombinant proteins were each expressed with a hexahistidine affinity tag at their N terminus and based on the requirement of pure antigen for use in subunit vaccine formulation, it was first investigated whether the recombinant proteins could indeed be purified by immobilized metal affinity chromatography (IMAC).

IMAC is based on the interactions between a transition metal ion (e.g. Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+}) immobilized on a matrix and specific amino acid side-chains. Among the different amino acids, histidine exhibits the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal. Following washing of the matrix, proteins containing a polyhistidine affinity tag can be eluted by either adjusting the pH or adding free imidazole to the elution buffer (Porath, 1992; Bornhorst and Falke, 2000).

The MagneHis™ Protein Purification System (Promega) was used to purify the recombinant proteins from IPTG-induced *E. coli* BL21-Gold(DE3) cultures. The soluble ETX0 protein was purified under non-denaturing (native) conditions, whereas the insoluble ETX1 and NC-ETX1 proteins were purified under denaturing conditions following solubilization with 8 M urea. To confirm successful purification, samples of the purified recombinant proteins were

subjected to Western blot analysis using an anti-polyhistidine antibody. The result, presented in Fig. 3.1, indicated that the antibody reacted with proteins corresponding with the expected sizes of the ETX1 (35 kDa) and NC-ETX1 (48 kDa) proteins, in addition to additional contaminating proteins. Interestingly, the antibody did not react with ETX0 purified from either the extracellular, periplasmic or cytoplasmic protein fractions.

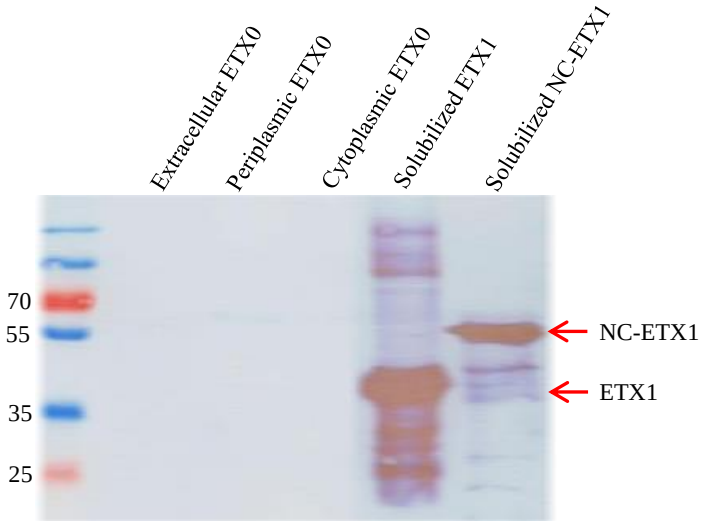


Fig. 3.1 Western blot analysis following expression and purification of the recombinant ETX0, ETX1 and NC-ETX1 proteins. Following expression of the respective proteins in IPTG-induced recombinant *E. coli* BL21-Gold(DE3) cultures, the ETX0 protein was purified by affinity chromatography from the extracellular, periplasmic and cytoplasmic protein fractions. The ETX1 and NC-ETX1 proteins were purified after solubilization with 8 M urea. The purified recombinant proteins were resolved by 12% SDS-PAGE and then subjected to Western blot analysis using an anti-polyhistidine antibody. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figure.

To determine whether the recombinant ETX0 protein was capable of binding to the resin, the experiment was repeated and a sample of the cytoplasmic protein fraction was analyzed together with samples obtained after sample loading and washing of the resin. Analysis of the Coomassie blue-stained SDS-polyacrylamide gel indicated that the recombinant ETX0 protein did not bind to the resin, as the protein was present in the supernatant following magnetic capturing of the MagneHis™ nickel particles (results not shown). These results suggested that the N-terminal hexahistidine tag of the recombinant ETX0 protein was either inaccessible for binding to the resin or that it may have been cleaved from the protein. The former seems unlikely, as the recombinant ETX0 protein was expressed in a soluble form that implies that it was correctly folded. Consequently, the amino acid sequence of the recombinant ETX0 protein was determined by mass spectrometry. The results indicated that the N-terminal sequence of the recombinant ETX0 protein was KEISNTVSNEMSKKASYDNVDTLIEKGR, indicating that the protein lacks the N-terminal 32-amino-acid signal peptide sequence. This result therefore suggests that the signal peptide sequence of ETX0 is cleaved by an *E. coli* signal peptidase, resulting in the removal of the N-terminal hexahistidine affinity tag, and thus explaining the inability of the recombinant ETX0 protein to bind to the affinity matrix. Furthermore, these results also indicated that the recombinant ETX1 and NC-ETX1 proteins, which lack the first nine amino acids of the signal peptide sequence, are not processed by the *E. coli* signal peptidase and can therefore be purified using IMAC.

Based on the above results, it was concluded that the recombinant ETX1 and NC-ETX1 proteins were solubilized sufficiently in 8 M urea to enable their purification by means of IMAC. However, due to removal of the N-terminal hexahistidine affinity tag, an alternative approach is required for purification of the recombinant ETX0 protein.

3.3.2 Purification of recombinant ETX0 by ion exchange chromatography

Based on the above results, indicating that intracellular processing of the recombinant ETX0 protein results in removal of the hexahistidine affinity tag, a different protein purification strategy was investigated. It has been reported previously that the epsilon toxin can be purified from *C. perfringens* Type D culture supernatants by making use of ion exchange chromatography (Orlans *et al.*, 1960; Thomson, 1963; Hauschild, 1965; Habeeb, 1969). Consequently, the recombinant ETX0 protein was purified from the cytoplasmic protein

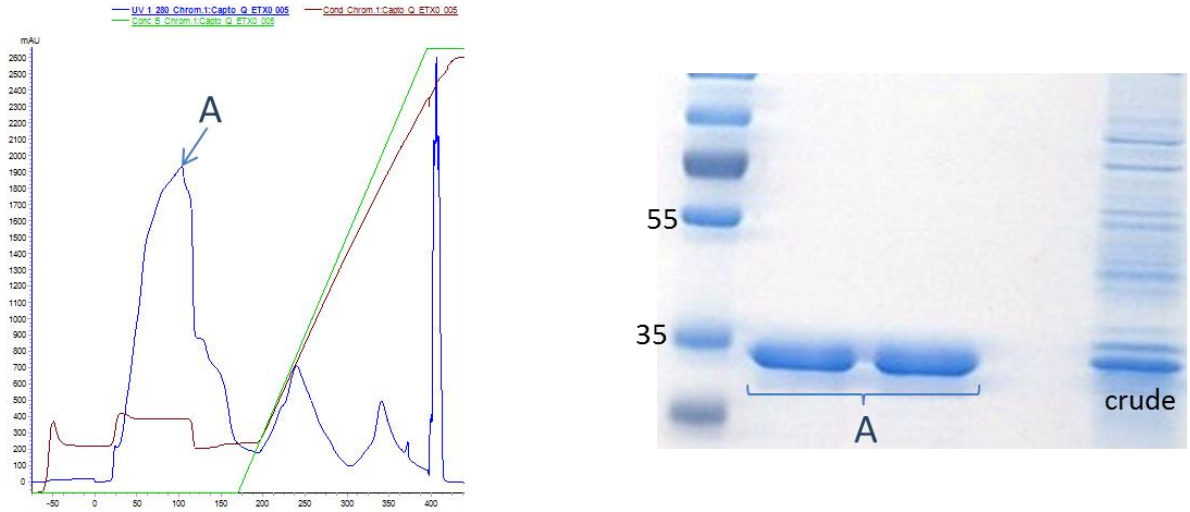
fraction of induced cultures by making use of Capto-Q as an anion exchange medium. For optimal results, it is recommended by the manufacturer of the resin that the pH of the buffer used for anion exchange should be 0.5-1 pH unit above the pI of the target protein (Capto-Q Product Information Sheet, GE HealthCare). Based on the predicted pI value of the recombinant ETX0 protein (8.75), diethanolamine (DEA) buffer with a pH of 9.5 was therefore selected for use in these experiments. Elution of the protein from the Capto-Q resin was monitored by determining the OD_{280} values of the collected fractions and the peak fractions were analyzed by SDS-PAGE.

In a first attempt at purifying the recombinant protein by ion exchange chromatography, 0.5 M DEA buffer was used. The results (Fig. 3.2a) indicated that following loading of the sample, there was a sharp increase in the OD_{280} values of fractions collected during washing of the resin, thus suggesting that the protein did not bind to the resin under these conditions. Nevertheless, SDS-PAGE analysis of the peak fractions indicated the presence of a highly purified protein corresponding in size with the 32-kDa ETX0 protein. The protein appeared to be purified to homogeneity as was evident by the lack of contaminating proteins, even when the SDS-polyacrylamide gel was overloaded with the samples.

Due to the unexpected nature of the above result, the methodology was critically reviewed and it was realized that the molarity of the DEA buffer was 10-fold higher than that recommended for use with the Capto-Q resin. Consequently, the experiment was repeated with 50 mM DEA buffer. The results (Fig. 3.2b) indicated that under these conditions, the recombinant ETX0 protein bound to the resin and could be eluted with the buffer, as was evidenced by a sharp increase in the OD_{280} values of fractions collected during the elution step. To verify that the recombinant ETX0 protein had been recovered and to assess its purity, aliquots of the peak fractions were analyzed by SDS-PAGE. Although analysis of the Coomassie blue-stained gel indicated the presence of the 32-kDa ETX0 protein, fractions containing the eluted ETX0 protein were not very pure since contaminating proteins were clearly visible on the stained gel.

Based on the greater purity of the recombinant ETX0 protein preparations obtained by making use of 0.5 M DEA buffer, all subsequent purifications of this recombinant protein were performed with this high molarity buffer. However, DEA is a potential skin irritant and has been reported to be toxic in a mouse study (Lessmann *et al.*, 2009; Craciunescu *et al.*, 2009). To allow for further evaluation of the purified ETX0 protein, the protein samples

(a)



(b)

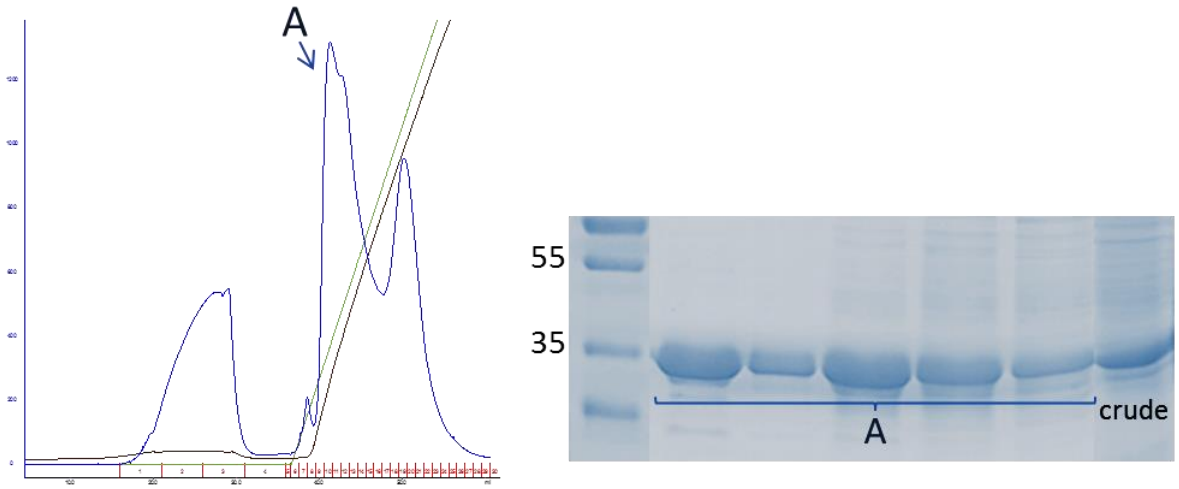


Fig. 3.2 Purification of the recombinant ETX0 protein by ion exchange chromatography. The cytoplasmic protein fraction of IPTG-induced recombinant bacterial cultures was loaded onto Capto-Q resin, washed and eluted in either (a) 0.5 M DEA buffer or (b) 50 mM DEA buffer. In each panel, both the chromatograph and corresponding Coomassie-blue stained SDS-polyacrylamide gel is provided. The samples were loaded at a flow rate of 2 ml/min, whereas washing of the resin and elution were performed at flow rate of 3 ml/min. In each chromatograph, the blue, brown and green graph lines represent the OD_{280} readings, the conductivity of the buffer and the concentration gradient of the elution buffer, respectively. The peak fractions, corresponding to A indicated in the chromatograph, were analyzed by 12% SDS-PAGE in the presence of a crude cytoplasmic protein extract. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figures.

were thus dialysed against PBS buffer, which is regarded as non-toxic and safe to inject into animals (Styszynski *et al.*, 2001). Using this protein purification strategy, the concentration of the purified ETX0 protein was determined to be 430 mg/l using a Bradford protein assay.

3.3.3 Purification of the recombinant NC-ETX1 and ETX1 proteins

The insoluble NC-ETX1 and ETX1 proteins were efficiently purified on a small scale with the MagneHis™ Protein Purification System, thus confirming that these proteins possessed a hexahistidine affinity tag and that it was accessible for binding to the resin. To purify the recombinant NC-ETX1 and ETX1 proteins on a large scale, affinity chromatography using two different reins, i.e. Ni-NTA and Ni-TED, was performed. Prior to purification, the insoluble inclusion bodies were solubilized with 8 M urea.

3.3.3.1 Purification of recombinant NC-ETX1 with Ni-NTA resin

Initially, the recombinant NC-ETX1 protein was purified by one-step affinity chromatography on a Ni-NTA resin. The solubilized protein sample was loaded onto the resin and then thoroughly washed with washing buffer containing urea. To allow for refolding of the denatured proteins while bound to the matrix, the resin was subsequently washed with buffer that lacked urea and the bound material was then eluted with imidazole.

The results (Fig. 3.3) indicated there was a sharp increase in the OD_{280} values of fractions collected after sample loading, while two small peaks could be observed in fractions collected during washing of the resin and elution of the bound proteins. Subsequent SDS-PAGE analysis of the respective peak fractions confirmed that some of the recombinant NC-ETX1 protein was washed from the resin after the sample loading and washing steps (Fig. 3.3b). Despite the small peak, analysis of the eluted material (peak C) revealed a predominant band of 48 kDa in the Coomassie blue-stained SDS-polyacrylamide gel. However, contaminants of high and low molecular mass were also detected on the stained gel. It should be noted that similar results could only be obtained when making use of freshly prepared Ni-NTA resin. The binding capacity of the resin was severely compromised upon regeneration and re-use of the resin, and resulted in progressively lower yields of eluted recombinant NC-ETX1 protein (Fig. 3.3c). Consequently, this resin was not considered to be a feasible option for

commercial purification of the recombinant NC-ETX1 protein and this approach was therefore discarded.

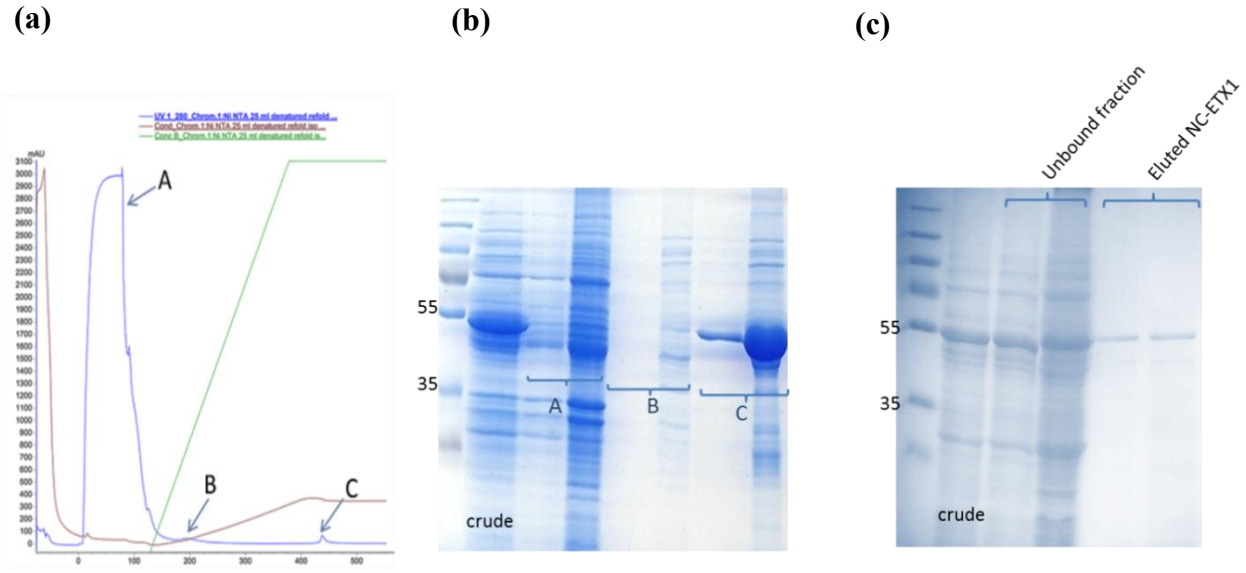


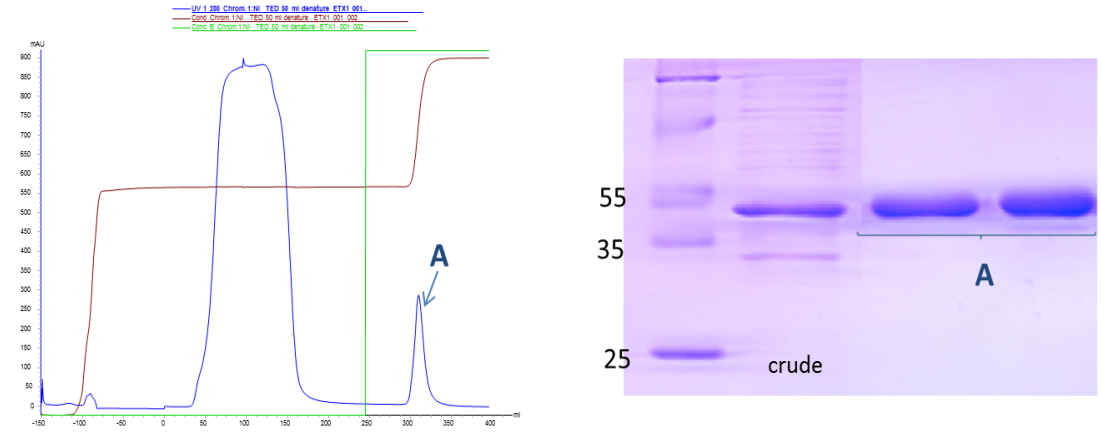
Fig. 3.3 Purification of the recombinant NC-ETX1 protein by Ni-NTA affinity chromatography. The recombinant NC-ETX1 protein was purified from inclusion bodies that had been solubilized in 8 M urea. The samples were loaded under denaturing conditions and allowed to refold into their native conformation while bound to the column, prior to elution with imidazole. Peak fractions identified in the chromatograph (a) were analyzed by 12% SDS-PAGE (b) in the presence of a crude cytoplasmic protein extract. Upon re-use of the Ni-NTA resin, a drastic decline in the amount of eluted NC-ETX1 protein was observed (c). The samples were loaded at a flow rate of 2 ml/min, whereas washing of the resin and elution were performed at flow rate of 3 ml/min. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figures.

3.3.3.2 Purification of recombinant ETX1 and NC-ETX1 with Ni-TED resin

Due to the rapid deterioration in the binding capacity of the Ni-NTA resin and the presence of several contaminating proteins present in the eluate, an alternative resin was evaluated for the purification of the recombinant ETX1 and NC-ETX1 proteins. For this purpose, a Ni-TED resin was selected. The chelating group of Ni-TED is based on TED (tris-carboxymethyl ethylene diamine), which is a strong pentadentate metal chelator. TED occupies five of the six binding sites in the coordination sphere of the Ni^{2+} ion, whereas the remaining site is available for protein binding. Compared to TED, nitrilotriacetic acid (NTA) has only four binding sites available for the Ni^{2+} ion, since the remaining two sites are available for protein binding. The additional chelation site of TED with Ni^{2+} is thought to mimimize metal leaching during purification and purportedly increases specificity for polyhistidine-tagged proteins (Varilova *et al.*, 2006).

To purify the recombinant ETX1 and NC-ETX1 proteins with the Ni-TED resin, a protocol similar to that described above was followed, except that the proteins were purified under denaturing conditions and subsequently refolded in buffer lacking urea. In contrast to results obtained using the Ni-NTA resin, for both the recombinant ETX1 and NC-ETX1 proteins, two distinct peaks were observed on the chromatographs. The OD_{280} values of fractions collected during the elution step were noticeably higher than that previously observed (Fig. 3.3), suggesting that unbound proteins were washed from the resin and bound proteins were eluted in the second peak (Fig. 3.4). SDS-PAGE analysis of peak fractions collected during the elution step indicated that the recombinant ETX1 (Fig. 3.4a) and NC-ETX1 (Fig. 3.4b) proteins were purified to near homogeneity, albeit that minor low molecular mass contaminating proteins could be observed in the purified NC-ETX1 protein preparation. Not only were the proteins purer, but their concentrations also appeared to be higher compared to the use of the Ni-NTA resin. In a final step, the elution buffer containing the urea denaturant was exchanged for TBS buffer by means of dialysis and the concentration of the purified recombinant proteins was determined. The results indicated that the yield of purified protein was 400 mg/l and 340 mg/l for the ETX1 and NC-ETX1 proteins, respectively.

(a)



(b)

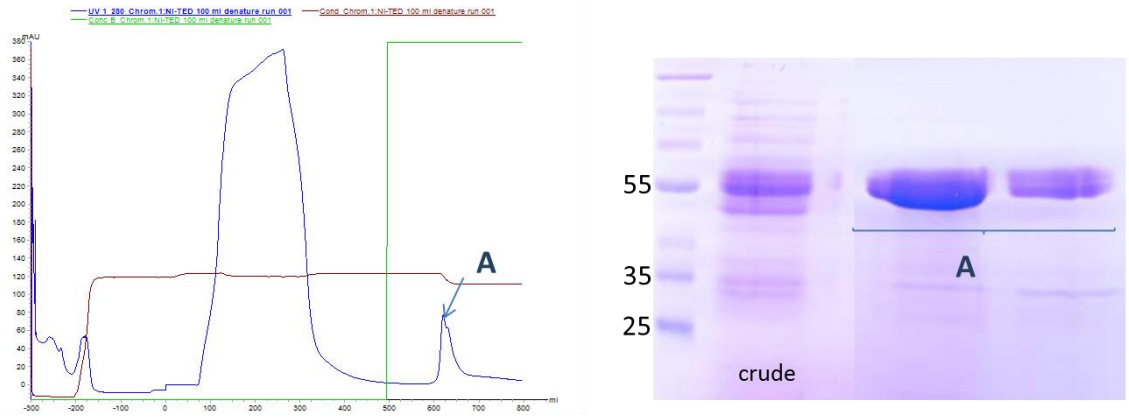


Fig. 3.4 Purification of the recombinant ETX1 and NC-ETX1 proteins by Ni-TED affinity chromatography. The recombinant ETX1 (a) and NC-ETX1 (b) proteins were purified from inclusion bodies that had been solubilized in 8 M urea. The purification was performed under denaturing conditions and the purified recombinant proteins were refolded in solution after affinity chromatography. In each panel, both the chromatograph and corresponding Coomassie-blue stained SDS-polyacrylamide gel is provided. The samples were loaded at a flow rate of 2 ml/min, whereas washing of the resin and elution were performed at flow rate of 3 ml/min. The peak fractions collected during the elution step, corresponding to A indicated in the chromatograph, were analyzed by 12% SDS-PAGE in the presence of a crude cytoplasmic protein extract. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figures.

3.3.4 Trypsin treatment of the recombinant ETX0 protein

The epsilon toxin is synthesized and secreted as a relatively inactive protoxin that is converted to the active toxin by proteolytic cleavage of the terminal peptides. Activation normally occurs in the gut of infected animals by the action of trypsin, α-chymotrypsin or *C. perfringens* lambda (λ) protease, but activation can also occur *in vitro* by making use of these enzymes (Bhown and Habeeb, 1977; Minami *et al.*, 1997).

To activate the ETX0 protoxin, non-purified ETX0 protein present in the cell-free culture supernatant was treated with trypsin. Subsequently, samples of the treated and untreated protein preparations were subjected to Western blot analysis using an anti-epsilon antibody. The result, presented in Fig. 3.5, indicated a shift in the banding pattern of the trypsin-treated ETX0 protein preparation. Compared to the untreated ETX0 protein, which banded at the expected position of 32 kDa, the trypsin-treated ETX0 protein was visibly smaller, indicating that the ETX0 protoxin was successfully digested. The lack of a 32-kDa protein in this preparation furthermore suggested that the protein was digested until completion.

3.3.5 Cytotoxic activity of recombinant proteins

The cytotoxic activity of purified recombinant epsilon proteins has been assessed by observing their effects on MDCK cells and it has been reported that the results of these assays correlates well with standard mouse lethality tests (Payne *et al.*, 1994; Petit *et al.*, 1997; Mathur *et al.*, 2010). Thus, in this study, the cytotoxicity of the different recombinant protein preparations was determined using a MDCK cell culture model, followed by staining of the cells with the metabolic indicator MTS. The MTS assay is based on the reduction of the yellow-coloured MTS by mitochondrial dehydrogenase enzymes of metabolically active cells to a soluble blue formazan product. The quantity of formazan can be determined spectrophotometrically by measuring the absorbance at 490 nm and is directly proportional to the number of viable cells in the culture (Cory *et al.*, 1991).

To investigate, MDCK cells were grown to confluence and then treated for 24 h with serial dilutions of purified ETX0, ETX1 and NC-ETX1, as well as with non-purified ETX0 and trypsin-treated ETX0. Thereafter, cell viability of the MDCK cells was measured by the MTT assay described above. Cells treated with Emetine, which irreversibly inhibits protein

synthesis in eukaryotic cells by binding to the 40S ribosomal subunit (Lietman, 1971), was included as a positive control in the assay. The IC_{50} values were determined, and the results are presented graphically in the Appendix to this dissertation and are summarized in Table 3.2.

In contrast to the purified recombinant ETX0 and ETX1 proteins, which were moderately toxic against the MDCK cells, the NC-ETX1 protein did not display cytotoxic activity to the cells. Treatment of the non-purified ETX0 protein, obtained from the culture supernatant, with trypsin resulted in a biologically active toxin that was very toxic against the MDCK cells compared to the untreated protein (1 200-fold more toxic) and was also more active than the Emetin positive control (661-fold more toxic). The buffers used in this study (PBS for ETX0, and TBS for ETX1 and NC-ETX1) displayed no cytotoxicity to the cells, indicating that the purified recombinant protein preparations should be safe for use in vaccine formulation and subsequent immunization of animals.

3.4 DISCUSSION

Since enterotoxemia caused by the *C. perfringens* Type D epsilon toxin is a pure toxemia, vaccination against the toxin is the most feasible preventive measure (Titball, 2009). Consequently, considerable effort has been directed to the development of recombinant subunit vaccines for prevention of Type D enterotoxemia (Oyston *et al.*, 1998; Lobato *et al.*, 2010; Souza *et al.*, 2010; Chandran *et al.*, 2010). These types of vaccines present a significant advantage in terms of safety considerations and the minimization of harmful side-effects, since the recombinant antigen can be purified to a high level and the composition of the subunit vaccine can therefore be defined clearly (Hansson *et al.*, 2000; Unnikrishnan *et al.*, 2012). Towards developing a safe recombinant subunit vaccine against enterotoxemia, the aim of this part of the study was thus to purify the recombinant ETX0, ETX1 and NC-ETX1 proteins in high amounts.

The Type D epsilon toxin has previously been expressed in *E. coli* with different affinity tags with the aim of facilitating its purification. Soluble expression of recombinant epsilon toxin in fusion with either a 26-kDa glutathione-S-transferase (GST) tag (Oyston *et al.*, 1998; Knapp *et al.*, 2009) or a 12-kDa thioredoxin (TrxA) tag (Zhao *et al.*, 2011) has been reported. Both these affinity tags are frequently used as a means to enhance the solubility of the

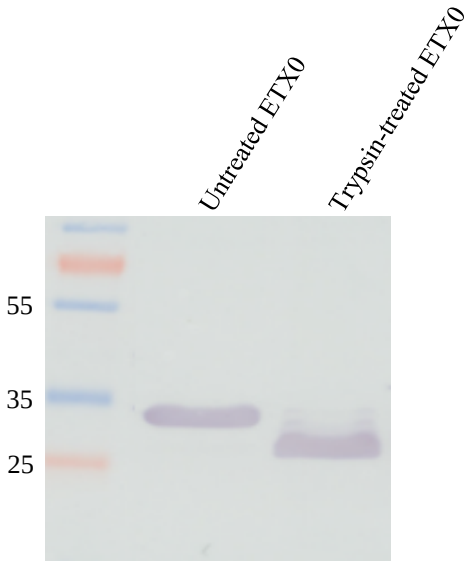


Fig. 3.5 Western blot analysis of the recombinant ETX0 protein following treatment with trypsin. The non-purified recombinant ETX0 protein from the cell-free culture supernatant was digested with trypsin *in vitro*. The untreated and treated ETX0 samples were resolved by 12% SDS-PAGE and then subjected to Western blot analysis using an anti-epsilon antibody. The sizes of the protein molecular mass marker, PageRuler™ Plus Pre-stained Protein Ladder (Fermentas), are indicated in kDa to the left of the figure.

Table 3.2 Cytotoxicity of the recombinant ETX0, ETX1 and NC-ETX1 proteins against MDCK cells

Sample No.	Sample name	Cytotoxicity	IC_{50} value*
1	Crude extracellular ETX0	Moderately toxic	0.1941
2	Trypsin-treated extracellular ETX0	Toxic	0.0001591
3	Purified ETX0	Moderately toxic	0.4549
4	Purified ETX1	Moderately toxic	0.3610
5	Purified NC-ETX1	Non-toxic	2.680
6	PBS	Non-toxic	32.84
7	TBS	Non-toxic	35.35
8	Emetine	Toxic	0.1053

* Cytoxicity was determined as the concentration of protein required to kill 50% of MDCK cells (IC_{50}).
Values ranging between 0.0001 and 0.005 are indicative of extreme toxicity, values ranging between 0.05 and 0.5 are indicative of moderate toxicity and values greater than 0.5 are considered as not toxic to the cells

expressed fusion proteins (Lichty *et al.*, 2005). However, unlike the GST affinity tag, TrxA does not facilitate purification on its own and a polyhistidine tag is typically used in conjunction with TrxA to enable purification of the target protein (Zhao *et al.*, 2011). In this study, the recombinant ETX0, ETX1 and NC-ETX1 proteins were expressed as fusion proteins with an N-terminal hexahistidine affinity tag. In contrast to the above affinity tags, the hexahistidine tag possesses several advantages, including its small size (0.84 kDa), low immunogenicity, and the purification of target proteins can be carried out under both native and denaturing conditions (Young *et al.*, 2012). The latter is possible because interaction of the polyhistidine tag with the resin does not require a specific conformation of the affinity tag.

Recombinant polyhistidine-tagged proteins are readily purified by immobilized metal affinity chromatography (IMAC) and various resins have been developed for this purpose (Bornhorst and Falke, 2000; Young *et al.*, 2012). The results of a small-scale experiment to determine whether the hexahistidine-tagged recombinant proteins could be purified by IMAC indicated that, in contrast to the ETX1 and NC-ETX1 proteins, the ETX0 protein could not be purified using this approach. Based on the results of Western blot analysis using an anti-polyhistidine antibody and mass spectrometry experiments, it was confirmed that the recombinant ETX0 protein lacks both the N-terminal affinity tag and epsilon toxin signal peptide sequence. This is indicative of intracellular processing of the signal peptide sequence by an *E. coli* signal peptidase and therefore explains why the soluble recombinant ETX0 protein could not be purified from either the cytoplasmic, periplasmic or extracellular protein fractions. Comparative analysis of the epsilon toxin signal peptide sequence indicated that it indeed possesses several structural features common of signal peptides used for the secretory production of recombinant proteins in *E. coli* (Fig. 3.6). These included the presence of a hydrophobic domain that is preceded by a positively-charged (hydrophilic) N-domain, as well as a C-domain that ends with an amino acid containing a small neutral side-chain, e.g. alanine, glycine or serine (Pugsley, 1993; Choi and Lee, 2004).

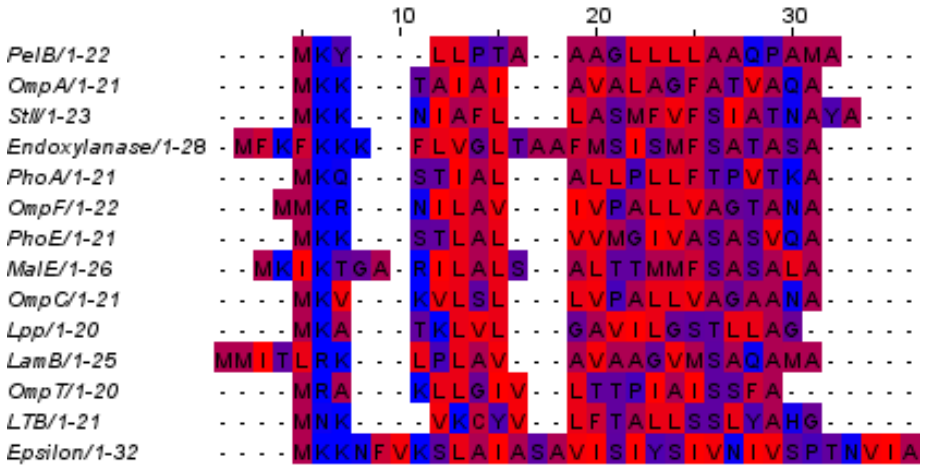


Fig. 3.6 Sequence alignment of the signal peptide sequence of the epsilon toxin with signal peptides used for secretory production of recombinant proteins in *E. coli*. The most hydrophobic amino acid residues are coloured in red and the most hydrophilic residues are coloured in blue. The multiple sequence alignment was performed with ClustalX (Larkin *et al*. 2007).

Why the recombinant ETX0 protein localized in the cell cytoplasm was also processed by the *E. coli* signal peptidase is not clear, but protein transport to the bacterial periplasm is a particularly complex process and not yet well understood (Danese and Sylhavy, 1998). However, similar to the results of this study, the cytoplasmic accumulation of recombinant penicillinase that had been cleaved in its signal sequence has been reported previously (Aono, 1992). Nevertheless, the recombinant ETX0 protein could be purified to homogeneity by ion exchange chromatography, albeit that 0.5 M DEA buffer was required for this purpose. The molarity of the DEA buffer is much higher than that recommended for use with the Capto-Q exchange medium (50 mM) and dialysis was required to exchange the potentially toxic DEA buffer with isotonic PBS buffer. Although this approach resulted in a high amount of purified protein (430 mg/l), it may not be a feasible approach for use in a commercial production system. Consequently, alternative approaches may be investigated in future. A potentially useful approach, which has been used to prepare highly purified *C. perfringens* Type D epsilon toxin from culture filtrates, relies on the use of DEAE-cellulose as the anion exchange medium (Worthington *et al.*, 1973).

The recombinant ETX1 and NC-ETX1 proteins, which lack the first nine amino acids of the N-terminal signal peptide sequence, were expressed as insoluble proteins in the cell cytoplasm, and both proteins were shown to possess the N-terminal hexahistidine affinity tag

as evidenced by their cross-reaction with an anti-polyhistidine antibody in Western blot analysis. A variety of methods has been published describing purification strategies for insoluble proteins, and typically involves solubilization of the insoluble protein followed by refolding of the protein into its native conformation (Rudolph and Lilie, 1996; Middelberg, 2002; Li *et al.*, 2004). Solubilization of the target protein from insoluble aggregates generally requires exposure to a strong chaotropic agent such as guanidium hydrochloride or urea. The use of urea as a denaturant is often preferable, since guanidinium hydrochloride precipitates in the presence of SDS and can thus interfere with subsequent SDS-PAGE analysis (Bornhorst and Falke, 2000). Urea mediates protein denaturation by disrupting hydrogen bonding, thereby leading to unfolding of the protein into a fully random conformation with all ionizable groups exposed to the solution (Herskovits *et al.*, 1970; Tsumoto *et al*., 2003). The solubilized denatured protein is almost always devoid of biological activity, which can be restored only if the protein can adopt a native or near-native configuration *in vitro*. In this investigation, the recombinant ETX1 and NC-ETX1 proteins were solubilized with 8 M urea and the NC-ETX1 protein could be purified by one-step affinity chromatography on Ni-NTA resin or by two-step affinity chromatography on Ni-TED resin. The two-step purification process, in which the NC-ETX1 protein was purified under denaturing conditions and then refolded into its active state by dialyzing away the urea denaturant, was also effective in purifying the ETX1 protein. The respective recombinant proteins were purified to near homogeneity, and the yield of purified proteins was determined to be 340 mg/l for NC-ETX1 and 400 mg/l for ETX1.

To evaluate the cytotoxicity of the purified recombinant ETX0, ETX1 and NC-ETX1 proteins, MDCK cells, which are sensitive to the epsilon toxin (Knight *et al.*, 1990; Payne *et al.*, 1994), were exposed to the respective protein preperations. In MDCK cells, the epsilon toxin induces cytoskeletal changes that results in loss of cell viability (Payne *et al.*, 1994; Petit *et al.*, 1997; Borrmann *et al.*, 2001; Petit *et al.*, 2003). The cytotoxicity of the epsilon toxin has been attributed to its ability to form membrane pores, thereby inducing the efflux of intracellular K^+ and the influx of Na^+ and Cl^- (Petit *et al.*, 2001; Chassin *et al.*, 2007). The results of the cytotoxicity assay indicated that the purified recombinant ETX0 and ETX1 proteins were moderately toxic to the MDCK cells, whereas the purified NC-ETX1 protein was not toxic (Table 3.2). However, it is interesting to note that the non-purified ETX0 was more toxic to the cells compared to the purified ETX0 protein (IC_{50} values of 0.19 and 0.45, respectively), suggesting the presence of toxigenic substances in the crude protein preparation

and thus furthermore substantiating the need to purify antigens prior to their formulation into vaccines. Moreover, activation of the non-purified ETX0 protein with trypsin resulted in increased toxicity towards the cells compared to the non-trypsined ETX0 protein control (ca. 1 200-fold). This is in agreement with microscopic observation of the cell monolayers, which indicated that the trypsin-treated ETX0 protein induced morphological changes such as rounding up of cells, as well as clumping and detachment of cells from the substratum more rapidly than the untreated ETX0 protein. The results are in agreement with previous results indicating that proteolytic activation can increase the specific toxicity of the epsilon prototoxin up to 1000-fold (Gill, 1987), and furthermore indicated that the recombinant ETX0 protein retained its biological activity.

In this study, the recombinant ETX1 and NC-ETX1 proteins were not treated with trypsin prior to their use in the cytotoxicity assay. This was based on a previous report indicating that a N-terminal truncated epsilon toxin, which is identical to the ETX1 protein described in this study, was considerably less toxic than the native toxin and subsequently allowed for its use in rabbits without further pre-treatments to reduce its toxicity (Souza *et al.*, 2010). Moreover, Goswami *et al.* (1996) reported that blocking the N-terminus of the epsilon toxin with a peptide, as was the case for the NC-ETX1 protein described in this study, resulted in a biologically inactive toxin. Since the recombinant ETX1 protein was therefore not treated with trypsin, which removes the C-terminal 22 amino acid residues of the epsilon toxin (Minami *et al.*, 1997), the results of the cytotoxicity assay may also indicate that cleavage of the C-terminal residues is essential for the biological activity of the epsilon toxin, whereas the removal of N-terminal amino acids seems to be of lesser importance. Moreover, the results obtained for the purified NC-ETX1 protein is particularly encouraging. Not only did fusing of the truncated flagellin protein to the N terminus of the ETX1 protein abolish its toxicity (and thus negates further steps to inactivate the recombinant protein), but it also has the added advantage of potentially providing a "built-in" adjuvant activity in this vaccine candidate. This is advantageous since simultaneous antigen delivery and TLR5 signaling via flagellin to the same antigen presenting cells can result in enhanced antigen uptake and presentation, as well as induction of humoral and cell-mediated immune responses (Cuadros *et al.*, 2004; Song *et al.*, 2008).

In conclusion, the recombinant ETX0, ETX1 and NC-ETX1 proteins could be purified by ion exchange or affinity chromatography procedures and resulted in excess of 300 mg/l of

purified proteins. Due to the high purity of these recombinant proteins, they can be used for further development and evaluation as recombinant subunit vaccine candidates against Type D enterotoxemia.

CHAPTER 4

Concluding Remarks

Towards the long-term aim of developing and evaluating a safe and efficacious recombinant subunit vaccine for enterotoxemia of livestock, caused by the epsilon toxin of *C. perfringens* Type D, the objectives of this study were essentially two-fold. The first objective was to clone and express the full-length epsilon toxin, as well as derivatives thereof in *E. coli*. These derivatives included a N-terminal truncated epsilon toxin, as well as fusion proteins comprised of the *B. halodurans* Alk36 flagellin protein and truncated epsilon toxin. The second objective was to purify the respective recombinant proteins and to evaluate their cytotoxicity in a mammalian cell culture model. The details of the results obtained in the course of achieving these objectives have been discussed in the individual chapters. The information that has evolved during this study will be summarized briefly and suggestions regarding future research will be made.

To avoid low expression levels of the epsilon toxin in *E. coli* (Hunter *et al.*, 1992; Goswami *et al.*, 1996; Souza *et al.*, 2010), an *E. coli* codon-optimized epsilon toxin gene (*etx*) was designed and chemically synthesized. The synthetic gene was subsequently used in the construction of recombinant bacterial expression vectors that contained the full-length *etx* gene (*etx*0), a truncated *etx* gene lacking the N-terminal nine amino acids (*etx*1), and a fusion gene (Δ*hag*::*etx*1) comprised of a truncated *B. halodurans* flagellin gene (Δ*hag*) and the *etx*1 gene. Interestingly, a *hag*::*etx*1 fusion gene, composed of the full-length *hag* gene and the *etx*1 gene, could not be obtained as it contained several nucleotide deletions in the region of a linker sequence that was introduced at the junction between the genes. The linker sequence, composed of Gly and Ser residues, enables the two proteins to keep their respective spatial conformations and biological function (Song *et al.*, 2008), and it has been reported not to interfere with antibody production (Song *et al.*, 2008; Liang *et al.*, 2012). No similar alterations as above were noted in the Δ*hag*::*etx*1 fusion gene, despite using a similar cloning strategy and an identical linker sequence. Moreover, construction of the *hag*::*etx*1 gene without the linker sequence solved the problem, but unfortunately this fusion gene contained a nucleotide substitution that introduced a premature stop codon. Although it is yet to be determined what the cause of the observed instability is, there appears to be a direct link between the use of the full-length *hag* sequence in combination with the linker sequence. Due to time constraints, this construct was excluded from further analyses. Nevertheless, the recombinant hexahistidine-tagged ETX0, ETX1 and NC-ETX1 proteins could be expressed successfully to high levels in the *E. coli* host. In addition to the use of a codon-optimized synthetic gene, the high yields of the recombinant proteins reported in this study can also be

attributed to the use of EnPresso™ as the production medium and optimization of both the cultivation temperature (30°C) and concentration of IPTG inducer.

The recombinant ETX0 protein was expressed in a soluble form, and could be detected in the cytoplasmic, periplasmic and extracellular protein fractions of induced *E. coli* cultures. However, the protein could not be purified by immobilized metal affinity chromatography (IMAC) due to intracellular processing of the epsilon toxin signal peptide sequence that resulted in the removal of the hexahistidine affinity tag. Consequently, the recombinant protein was purified using ion exchange chromatography. To enable purification of the recombinant ETX0 protein by IMAC, the gene may in future be cloned into a pET bacterial expression vector that is capable of expressing the recombinant protein with a C-terminal hexahistidine tag. The accumulation of recombinant ETX0 protein in the cell periplasm and culture medium could potentially present significant advantages with regards to purification of this recombinant protein. Advantages associated with secretion of the protein to the culture medium include low levels of proteolysis, simpler purification strategies and improved protein folding (Mergulhao *et al.*, 2005; Wong *et al.*, 2012). Moreover, considering that the periplasm contains only 4% of the total cell protein (Pugsley and Schwartz, 1985), localization of the protein in the periplasm may serve to concentrate the target protein in this cellular compartment and may make its purification therefore less tedious. In addition, the oxidizing environment of the periplasm is also reported to facilitate proper folding of proteins (Missiakas and Raina, 1997). To exploit these advantages, the use of signal peptides that have been reported to facilitate efficient secretion of recombinant proteins from *E. coli* can be considered. These include the signal sequences of the heat-labile enterotoxin (Jobling *et al.*, 1997), alkaline phosphatase (Zhang *et al.*, 2007) and OmpA (Ray *et al.*, 2002). Alternatively, the epsilon toxin signal peptide sequence can be genetically modified to more closely represent signal sequences used by the Gram-negative *E. coli* bacterium in order to improve secretory production of the recombinant ETX0 protein (Choi and Lee, 2004; Low *et al.*, 2013).

In contrast to the ETX0 protein, both the recombinant ETX1 and NC-ETX1 proteins were expressed as insoluble proteins in the cell cytoplasm and they could be purified to near homogeneity under denaturing conditions by IMAC using a Ni-TED resin. Despite optimization of various parameters (cultivation medium, IPTG concentration and temperature), none of these resulted in the increased production of soluble protein. Thus,

alternative strategies may in future be investigated as a means to express the ETX1 and NC-ETX1 proteins in a soluble form. Rather than using a strong inducible promoter such as the bacteriophage T7 RNA polymerase promoter used in this study, the usefulness of cold-responsive promoters may in future be investigated since they can facilitate expression of heterologous genes at much reduced temperatures (Baneyx and Mujacic, 2003). For example, the promoter of the *E. coli* major cold shock gene *cspA* has been demonstrated to be active at about 15 to 20°C (Vasina and Baneyx, 1996b). The rationale behind the use of cold-responsive promoters for gene expression is based on the proposition that the rate of protein folding will be only slightly affected at lower temperatures, whereas the rates of transcription and translation, being biochemical reactions, will be decreased substantially. This, in turn, will provide sufficient time for protein refolding and thus avoid the formation of inactive protein aggregates, i.e. inclusion bodies (Baneyx and Mujacic, 2003).

Alternatively, simultaneous overexpression of chaperone-encoding genes and the recombinant ETX1 and NC-ETX1 proteins may also be investigated as a means to prevent or reduce inclusion body formation, thereby improving the solubility of these recombinant proteins (Baneyx and Palumbo, 2003; Kolaj *et al.*, 2009; Frances and Page, 2010). Soluble recombinant protein expression has been stimulated by the co-overexpression of the GroEL-GroES and DnaK-DnaJ-GrpE chaperone systems. The DnaK/DnaJ chaperonin system, members of the Hsp70 chaperone family, binds to hydrophobic segments of the unfolded polypeptide in order to maintain solubility and prevent aggregation, and dissociation of DnaK and DnaJ from the polypeptide is facilitated by protein GrpE (Caspers *et al.*, 1994; Perez-Perez *et al.*, 1995; Lee *et al.*, 2004). In contrast, the GroEL/GroES chaperonin system, members of the Hsp60 and Hsp10 chaperone families, respectively, mediates solubilization of proteins by binding to misfolded polypeptides and allows them to refold when released (Lee and Olins, 1992; Kohda *et al.*, 2002; Park *et al.*, 2004; Heo *et al.*, 2006).

The toxicity of the purified recombinant ETX0, ETX1 and NC-ETX1 proteins towards Madin-Darby canine kidney cells (MDCK) cells was also assayed in this study. In contrast to the ETX0 and ETX1 proteins, which were moderately toxic to the cells, the NC-ETX1 protein was not toxic. These results indicate that before the recombinant ETX0 and ETX1 proteins can be used as antigens in vaccine formulation, they would have to be inactivated by treatment with formaldehyde. Once complete inactivation and the lack of residual toxicity have been established, then only can they be used in animal trials to determine their protective

efficacy. In contrast, the lack of toxicity displayed by the recombinant NC-ETX1 protein suggests that it may be used without further pre-treatment steps and thus represents a cost saving benefit. Moreover, in contrast to the recombinant ETX0 and ETX1 proteins that will require the addition of an adjuvant in the vaccine formulation, the NC-ETX1 fusion protein already contains a "potential" adjuvant in the form of the truncated flagellin protein.

It has been reported that flagellin has adjuvant activity capable of inducing or boosting adaptive immune responses by specifically binding to Toll-like receptor 5 (TLR5) (Cuadros *et al.*, 2004; Honko and Mizel, 2005; Kawai and Akira, 2010). Therefore, it is imperative that the TLR5-specific signalling activity of the recombinant NC-ETX1 protein be evaluated in future studies. This may be performed by treating TLR5-negative and -positive RAW264.7 cells (McDonald *et al.*, 2007; Huleatt *et al.*, 2007; Mizel *et al.*, 2009) with the NC-ETX1 fusion protein, and then determining the amount of TNF-α produced in these cells. In addition, the ability of the NC-ETX1 protein to induce an antigen-specific humoral immune response, as well as a cellular-mediated immune response in immunized animals should also be established. This may be done by assaying for anti-epsilon toxin IgG antibodies by ELISA and by performing T cell lymphocyte proliferation assays, respectively (Cuadros *et al.*, 2004; Karam *et al.*, 2013). Once these assays have been performed and if found to elicit these immune responses, then the ability of the recombinant NC-ETX1 protein to protect animals against challenge with the epsilon toxin can be investigated.

Due to time constraints the above types of studies could not be performed and the recombinant proteins could therefore not be evaluated for their protective efficacy in animal trials. Nevertheless, in this study the high-level expression and purification of three vaccine candidates against Type D enterotoxemia was described. As potential veterinary vaccines for livestock animals, they would have several advantages over the currently available commercial vaccines. The recombinant vaccines would be well-defined and standardized and thus the immunogenicity of the vaccine would be more uniform than the current vaccines, which are produced by formaldehyde treatment of bacterial culture filtrates or whole-cell cultures. In addition, they would be easier and cheaper to produce since the need to anaerobically grow large volumes of the pathogen would be bypassed and instead relies on the culture of a laboratory strain of *E. coli*. Consequently, further evaluation of these recombinant proteins as potential vaccine candidates is recommended.

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APPENDICES

A1: Nucleotide sequence and deduced amino acid sequence of the *E. coli* codon-optimized *etx*0 gene. The signal peptide sequence is indicated in red.

```
1          ATGAAAAAAAACCTGGTTAAATCTCTGGCGATCGCGTCTGCGGTTATCTCTATCTACTCT
1           M  K  K  N  L  V  K  S  L  A  I  A  S  A  V  I  S  I  Y  S

61         ATCGTTAACATCGTTTCTCCGACCAACGTTATCGCGAAAGAAATCTCTAACACCGTTTCT
21          I  V  N  I  V  S  P  T  N  V  I  A  K  E  I  S  N  T  V  S

121        AACGAAATGTCTAAAAAAGCGTCTTACGACAACGTTGACACCCTGATCGAAAAAGGTCGT
41          N  E  M  S  K  K  A  S  Y  D  N  V  D  T  L  I  E  K  G  R

181        TACAACACCAAATACAACTACCTGAAACGTATGGAAAAATACTACCCGAACGCGATGGCG
61          Y  N  T  K  Y  N  Y  L  K  R  M  E  K  Y  Y  P  N  A  M  A

241        TACTTCGACAAAGTTACCATCAACCCGCAGGGTAACGACTTCTACATCAACAACCCGAAA
81          Y  F  D  K  V  T  I  N  P  Q  G  N  D  F  Y  I  N  N  P  K

301        GTTGAACTGGACGGTGAACCGTCTATGAACTACCTGGAAGACGTTTACGTTGGTAAAGCG
101         V  E  L  D  G  E  P  S  M  N  Y  L  E  D  V  Y  V  G  K  A

361        CTGCTGACCAACGACACCCAGCAGGAACAGAAACTGAAATCTCAGTCTTTCACCTGCAAA
121         L  L  T  N  D  T  Q  Q  E  Q  K  L  K  S  Q  S  F  T  C  K

421        AACACCGACACCGTTACCGCGACCACCACCCACACCGTTGGTACCTCTATCCAGGCGACC
141         N  T  D  T  V  T  A  T  T  T  H  T  V  G  T  S  I  Q  A  T

481        GCGAAATTCACCGTTCCGTTCAACGAAACCGGTGTTTCTCTGACCACCTCTTACTCTTTC
161         A  K  F  T  V  P  F  N  E  T  G  V  S  L  T  T  S  Y  S  F

541        GCGAACACCAACACCAACACCAACTCTAAAGAAATCACCCACAACGTTCCGTCTCAGGAC
181         A  N  T  N  T  N  T  N  S  K  E  I  T  H  N  V  P  S  Q  D

601        ATCCTGGTTCCGGCGAACACCACCGTTGAAGTTATCGCGTACCTGAAAAAAGTTAACGTT
201         I  L  V  P  A  N  T  T  V  E  V  I  A  Y  L  K  K  V  N  V

661        AAAGGTAACGTTAAACTGGTTGGTCAGGTTTCTGGTTCTGAATGGGGTGAAATCCCGTCT
221         K  G  N  V  K  L  V  G  Q  V  S  G  S  E  W  G  E  I  P  S

721        TACCTGGCGTTCCCGCGTGACGGTTACAAATTCTCTCTGTCTGACACCGTTAACAAATCT
241         Y  L  A  F  P  R  D  G  Y  K  F  S  L  S  D  T  V  N  K  S

781        GACCTGAACGAAGACGGTACCATCAACATCAACGGTAAAGGTAACTACTCTGCGGTTATG
261         D  L  N  E  D  G  T  I  N  I  N  G  K  G  N  Y  S  A  V  M

841        GGTGACGAACTGATCGTTAAAGTTCGTAACCTGAACACCAACAACGTTCAGGAATACGTT
281         G  D  E  L  I  V  K  V  R  N  L  N  T  N  N  V  Q  E  Y  V

901        ATCCCGGTTGACAAAAAAGAAAAATCTAACGACTCTAACATCGTTAAATACCGTTCTCTG
301         I  P  V  D  K  K  E  K  S  N  D  S  N  I  V  K  Y  R  S  L

961        TACATCAAAGCGCCGGGTATCAAATAA
321         Y  I  K  A  P  G  I  K  *
```

A2: Nucleotide sequence and deduced amino acid sequence of the *etx*1 gene. The restriction enzyme sites used for cloning purposes are indicated in bold, and the truncated signal peptide sequence, lacking the first nine amino acids, is indicated in red.

```
1          GGATCCGCGATCGCGTCTGCGGTTATCTCTATCTACTCTATCGTTAACATCGTTTCTCCG
1           G  S  A  I  A  S  A  V  I  S  I  Y  S  I  V  N  I  V  S  P

61         ACCAACGTTATCGCGAAAGAAATCTCTAACACCGTTTCTAACGAAATGTCTAAAAAAGCG
21          T  N  V  I  A  K  E  I  S  N  T  V  S  N  E  M  S  K  K  A

121        TCTTACGACAACGTTGACACCCTGATCGAAAAAGGTCGTTACAACACCAAATACAACTAC
41          S  Y  D  N  V  D  T  L  I  E  K  G  R  Y  N  T  K  Y  N  Y

181        CTGAAACGTATGGAAAAATACTACCCGAACGCGATGGCGTACTTCGACAAAGTTACCATC
61          L  K  R  M  E  K  Y  Y  P  N  A  M  A  Y  F  D  K  V  T  I

241        AACCCGCAGGGTAACGACTTCTACATCAACAACCCGAAAGTTGAACTGGACGGTGAACCG
81          N  P  Q  G  N  D  F  Y  I  N  N  P  K  V  E  L  D  G  E  P

301        TCTATGAACTACCTGGAAGACGTTTACGTTGGTAAAGCGCTGCTGACCAACGACACCCAG
101         S  M  N  Y  L  E  D  V  Y  V  G  K  A  L  L  T  N  D  T  Q

361        CAGGAACAGAAACTGAAATCTCAGTCTTTCACCTGCAAAAACACCGACACCGTTACCGCG
121         Q  E  Q  K  L  K  S  Q  S  F  T  C  K  N  T  D  T  V  T  A

421        ACCACCACCCACACCGTTGGTACCTCTATCCAGGCGACCGCGAAATTCACCGTTCCGTTC
141         T  T  T  H  T  V  G  T  S  I  Q  A  T  A  K  F  T  V  P  F

481        AACGAAACCGGTGTTTCTCTGACCACCTCTTACTCTTTCGCGAACACCAACACCAACACC
161         N  E  T  G  V  S  L  T  T  S  Y  S  F  A  N  T  N  T  N  T

541        AACTCTAAAGAAATCACCCACAACGTTCCGTCTCAGGACATCCTGGTTCCGGCGAACACC
181         N  S  K  E  I  T  H  N  V  P  S  Q  D  I  L  V  P  A  N  T

601        ACCGTTGAAGTTATCGCGTACCTGAAAAAAGTTAACGTTAAAGGTAACGTTAAACTGGTT
201         T  V  E  V  I  A  Y  L  K  K  V  N  V  K  G  N  V  K  L  V

661        GGTCAGGTTTCTGGTTCTGAATGGGGTGAAATCCCGTCTTACCTGGCGTTCCCGCGTGAC
221         G  Q  V  S  G  S  E  W  G  E  I  P  S  Y  L  A  F  P  R  D

721        GGTTACAAATTCTCTCTGTCTGACACCGTTAACAAATCTGACCTGAACGAAGACGGTACC
241         G  Y  K  F  S  L  S  D  T  V  N  K  S  D  L  N  E  D  G  T

781        ATCAACATCAACGGTAAAGGTAACTACTCTGCGGTTATGGGTGACGAACTGATCGTTAAA
261         I  N  I  N  G  K  G  N  Y  S  A  V  M  G  D  E  L  I  V  K

841        GTTCGTAACCTGAACACCAACAACGTTCAGGAATACGTTATCCCGGTTGACAAAAAAGAA
281         V  R  N  L  N  T  N  N  V  Q  E  Y  V  I  P  V  D  K  K  E

901        AAATCTAACGACTCTAACATCGTTAAATACCGTTCTCTGTACATCAAAGCGCCGGGTATC
301         K  S  N  D  S  N  I  V  K  Y  R  S  L  Y  I  K  A  P  G  I

961        AAATAAAAGCTT
321         K  *
```

A3: Nucleotide sequence and deduced amino acid sequence of the Δ*hag::etx*1 gene, constructed by fusing a truncated *hag* gene (indicated by white arrows) from *B. halodurans* Alk36 in-frame with the *etx*1 gene (indicated by black arrows). The linker sequence at the junction site is indicated in red, and the restriction enzyme sites used during construction of the fusion gene are indicated in bold.

```
1          GGATCCATTATCAATCACAATTTACCAGCAATGAATGCGCATCGTAACATGGGTATCAAT
1           G  S  I  I  N  H  N  L  P  A  M  N  A  H  R  N  M  G  I  N

61         CTCAACCAAGGTCAAAAAGCGATGGAGAAGCTTTCTTCAGGTCTTCGCATTAACCGTGCA
21          L  N  Q  G  Q  K  A  M  E  K  L  S  S  G  L  R  I  N  R  A

121        GGAGACGATGCTGCAGGTGTCGACCGCTCTTACCTAGGAGCTATGCAAAACCGCCTAGAG
41          G  D  D  A  A  G  V  D  R  S  Y  L  G  A  M  Q  N  R  L  E

181        CATACAATCAAAAACCTTGATAATGCTTCTGAAAACCTTCAAGCTGCTGAGTCTCGTATC
61          H  T  I  K  N  L  D  N  A  S  E  N  L  Q  A  A  E  S  R  I

241        CGTGACGTAGACATGGCGAAAGAAATGATGGAGTTCACAAGAACAAACATCTTAAACCAA
81          R  D  V  D  M  A  K  E  M  M  E  F  T  R  T  N  I  L  N  Q

301        GCGTCTCAAGCGATGCTTGCTCAAGCAAACCAACAGCCACAAGCTGTATTACAATTACTT
101         A  S  Q  A  M  L  A  Q  A  N  Q  Q  P  Q  A  V  L  Q  L  L

361        CGTCTCGAGTCTGGTTCTGGTTCTGGTTCTGCGATCGCGTCTGCGGTTATCTCTATCTAC
121         R  L  E  S  G  S  G  S  G  S  A  I  A  S  A  V  I  S  I  Y

421        TCTATCGTTAACATCGTTTCTCCGACCAACGTTATCGCGAAAGAAATCTCTAACACCGTT
141         S  I  V  N  I  V  S  P  T  N  V  I  A  K  E  I  S  N  T  V

481        TCTAACGAAATGTCTAAAAAAGCGTCTTACGACAACGTTGACACCCTGATCGAAAAAGGT
161         S  N  E  M  S  K  K  A  S  Y  D  N  V  D  T  L  I  E  K  G

541        CGTTACAACACCAAATACAACTACCTGAAACGTATGGAAAAATACTACCCGAACGCGATG
181         R  Y  N  T  K  Y  N  Y  L  K  R  M  E  K  Y  Y  P  N  A  M

601        GCGTACTTCGACAAAGTTACCATCAACCCGCAGGGTAACGACTTCTACATCAACAACCCG
201         A  Y  F  D  K  V  T  I  N  P  Q  G  N  D  F  Y  I  N  N  P

661        AAAGTTGAACTGGACGGTGAACCGTCTATGAACTACCTGGAAGACGTTTACGTTGGTAAA
221         K  V  E  L  D  G  E  P  S  M  N  Y  L  E  D  V  Y  V  G  K

721        GCGCTGCTGACCAACGACACCCAGCAGGAACAGAAACTGAAATCTCAGTCTTTCACCTGC
241         A  L  L  T  N  D  T  Q  Q  E  Q  K  L  K  S  Q  S  F  T  C

781        AAAAACACCGACACCGTTACCGCGACCACCACCCACACCGTTGGTACCTCTATCCAGGCG
261         K  N  T  D  T  V  T  A  T  T  T  H  T  V  G  T  S  I  Q  A

841        ACCGCGAAATTCACCGTTCCGTTCAACGAAACCGGTGTTTCTCTGACCACCTCTTACTCT
281         T  A  K  F  T  V  P  F  N  E  T  G  V  S  L  T  T  S  Y  S

901        TTCGCGAACACCAACACCAACACCAACTCTAAAGAAATCACCCACAACGTTCCGTCTCAG
301         F  A  N  T  N  T  N  T  N  S  K  E  I  T  H  N  V  P  S  Q

961        GACATCCTGGTTCCGGCGAACACCACCGTTGAAGTTATCGCGTACCTGAAAAAAGTTAAC
321         D  I  L  V  P  A  N  T  T  V  E  V  I  A  Y  L  K  K  V  N

1021       GTTAAAGGTAACGTTAAACTGGTTGGTCAGGTTTCTGGTTCTGAATGGGGTGAAATCCCG
341         V  K  G  N  V  K  L  V  G  Q  V  S  G  S  E  W  G  E  I  P
```

```
1081      TCTTACCTGGCGTTCCCGCGTGACGGTTACAAATTCTCTCTGTCTGACACCGTTAACAAA
361        S  Y  L  A  F  P  R  D  G  Y  K  F  S  L  S  D  T  V  N  K

1141      TCTGACCTGAACGAAGACGGTACCATCAACATCAACGGTAAAGGTAACTACTCTGCGGTT
381        S  D  L  N  E  D  G  T  I  N  I  N  G  K  G  N  Y  S  A  V

1201      ATGGGTGACGAACTGATCGTTAAAGTTCGTAACCTGAACACCAACAACGTTCAGGAATAC
401        M  G  D  E  L  I  V  K  V  R  N  L  N  T  N  N  V  Q  E  Y

1261      GTTATCCCGGTTGACAAAAAAGAAAAATCTAACGACTCTAACATCGTTAAATACCGTTCT
421        V  I  P  V  D  K  K  E  K  S  N  D  S  N  I  V  K  Y  R  S

1321      CTGTACATCAAAGCGCCGGGTATCAAATAAAAGCTT
441        L  Y  I  K  A  P  G  I  K  *
```

A4: Cytotoxicity assays.

Graphical representation of the half maximal inhibitory concentration (IC_{50}) of the recombinant ETX0, ETX1 and NC-ETX1 proteins towards MDCK cells. The percentage of viable MDCK cells was plotted against the logarithm concentration of each sample. The samples were subjected to 10-fold serial dilutions before being added to the cell monolayers. The crude extracellular ETX0 protein (CEE) was also treated with trypsin and assayed similarly. Emetine was included as a positive control. Phosphate-buffered saline (PBS) and Tris-buffered saline (TBS), used in buffer exchanges, were also included to assess their toxicity towards the cells.